EVALUATION OF A PROPRIETARY SLOW-RELEASE OXYTOCIN THERAPY AND RETURN OF THE LUTEOLYTIC MECHANISM IN MARES

by

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ABSTRACT

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Utah State University, 2019

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Prolonging function of the corpus luteum (CL) is a method of suppressing estrus that maintains secretion of endogenous progesterone to keep mares out of heat naturally. The use of oxytocin to prolong CL function is becoming more popular. In these therapies, upregulation of cyclooxygenase-2 is inhibited, which impairs prostaglandin F2α (PGF2α) production. Intramuscular (IM) administration of 60 IU of oxytocin once daily from 7 to 14 days after ovulation is currently the most common treatment protocol. Although that protocol is efficacious in ≥70% of treated mares, the need for daily administration is a drawback to its use. A proprietary slow-release oxytocin formulation (SR-OT) with a two-injection protocol to prolong CL function was evaluated in the first experiment. Mares were examined to determine the day of ovulation (day 0) and then randomly assigned either to a non-treated control group or an SR-OT treatment group (n = 8 mares/group). Mares in the treated group received 1.0 mL of SR-OT containing 2,400 IU

oxytocin IM once on Day 7 and again on Day 10 after ovulation. Jugular blood samples were collected on day 0 and then every M, W, and F through day 50. Serum progesterone concentrations were evaluated to assess CL function, which was prolonged in 0/8 (0%) control mares and 6/8 (75%) of the SR-OT treated mares ($p < 0.01$). In a second study, the ability of the endometrium to synthesize PGF2α was evaluated in mares in a state of prolonged CL function. Mares were designated into groups 50-59, 60-69, or 70-79 days post-ovulation (50s, 60s, 70s) and 14-day post-ovulation controls. PGF2α synthesis was evaluated by measurement of PGFM in response to a single 10 IU intravenous oxytocin bolus (0 minutes). Blood samples were collected serially from 30 minutes before until 120 minutes after oxytocin administration. The PGFM response was significantly higher in the 70s versus the 50s and 70s versus the 60s groups ($p < 0.001$; $p < 0.02$, respectively); and there was no significant difference between the 70s group and the control group ($p > 0.36$). Luteal function was maintained after oxytocin administration in 4/4, 3/4, and 0/3 mares in the 50s, 60s, and 70s groups, respectively.

(96 pages)

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Prolonging function of the corpus luteum (CL) is a method of suppressing estrus that maintains secretion of endogenous progesterone to keep mares out of heat naturally. The use of oxytocin to prolong CL function is becoming more popular. In these therapies, upregulation of cyclooxygenase-2 is inhibited, which impairs prostaglandin F2α (PGF2α) production. Intramuscular (IM) administration of 60 IU of oxytocin once daily from 7 to 14 days after ovulation is currently the most common treatment protocol. Although that protocol is efficacious in ≥70% of treated mares, the need for daily administration is a drawback to its use. A proprietary slow-release oxytocin formulation (SR-OT) with a two-injection protocol to prolong CL function was evaluated in the first experiment. Mares were examined to determine the day of ovulation (day 0) and then randomly assigned either to a non-treated control group or an SR-OT treatment group (n = 8 mares/group). Mares in the treated group received 1.0 mL of SR-OT containing 2,400 IU oxytocin IM once on Day 7 and again on Day 10 after ovulation. Jugular blood samples were collected on day 0 and then every M, W, and F continuously. Serum progesterone concentrations were evaluated to assess CL function, which was prolonged in 0/8 (0%) control mares and 6/8 (75%) of the SR-OT treated mares ($p < 0.01$). In a second study, the ability of the endometrium to synthesize PGF2α was evaluated in mares in a state of

prolonged CL function. Mares were designated into groups 50-59, 60-69, or 70-79 days post-ovulation (50s, 60s, 70s) and 14-day post-ovulation controls. PGF2α synthesis was evaluated by measurement of a prostaglandin metabolite in response to a single 10 IU intravenous oxytocin bolus (0 minutes). Blood samples were collected serially from 30 minutes before until 120 minutes after oxytocin administration. The metabolite response was significantly higher in the 70s versus the 50s and 70s versus the 60s groups ($p < 0.001$; $p < 0.02$, respectively); and there was no significant difference between the 70s group and the control group ($P > 0.36$). Luteal function was maintained after oxytocin administration in 4/4, 3/4, and 0/3 mares in the 50s, 60s, and 70s groups, respectively. Collectively, these results indicate that the luteolytic mechanism returns approximately 70 days into the period of prolonged CL function.

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I would like to thank Drs. Dirk Vanderwall and Holly Mason for their unwavering support. Through my time as a graduate student I have truly enjoyed learning about so much more than what this research and review provides. I would also like to express my gratitude to Dr. Clay Isom, who completed my committee and brought so much comparative insight to the table. The completion of my thesis would not have been possible without the love and support from my family and friends. Thank you, for the examples of hard work and the continuing encouragement.

Brendan Sarnecky

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LIST OF ABBREVIATIONS

AA = arachidonic acid

ANOVA = analysis of variance

AUC = area under the curve

CIDR = controlled intravaginal/internal drug release

CL = corpus luteum

cm = centimeter

COX-1 = cyclooxygenase 1

COX-2 = cyclooxygenase 2

eCG = equine chorionic gonadotropin

F = Friday

FDA = Food and Drug Administration

FSH = follicle stimulating hormone

g = gram

GnRH = gonadotropin releasing hormone

hCG = human chorionic gonadotropin

HPO = hypothalamic-pituitary-ovarian

IACUC = Institutional Animal Care and Use Committee

IM = intramuscular

IUD = intrauterine device

IV = intravenous

LH = luteinizing hormone

M = Monday

mg/kg = milligrams per kilogram

mm = millimeter

ng/mL = nanograms per milliliter

MPA = medroxyprogesterone actetate

pg/mL = picograms per milliliter

PGE2 = prostaglandin E2

PGFM = 13,14-dihydro-15-keto prostaglandin F2α

PGFS = prostaglandin F synthase

PGF2α = prostaglandin F2α

PGG2 = prostaglandin G2

PGH2 = prostaglandin H2

PLA2 = phospholipase A2

µl = microliter

SD = standard deviation

SR-OT = slow-release oxytocin

W = Wednesday

CHAPTER I

INTRODUCTION

In all domestic animals, it is a goal of the owner, producer, trainer, handler, or veterinarian to manage behaviors with techniques that provide the greatest opportunity for success. In the management of livestock, it is understood that stress associated with handling can have an effect on production. The need to address behavioral concerns has even given rise to behavior as a veterinary medicine specialty (American College of Veterinary Behaviorists). In companion animal species, household pets are often treated as family members. In the equine industry, trainability, safety, and performance can be greatly impacted by behavior. The expression of behavioral estrus in mares, can have a profoundly negative influence on performance [1]. It is common for horse owners to approach veterinarians seeking of help to manage these behaviors [2].

There are many estrus suppression methods which therapeutically address behaviors associated with the estrous cycle. The most popular methods of estrus suppression include the use of exogenous progestins, extending the functional span of the corpus luteum (CL), suppressing follicular activity, and ovariectomy [3]. The most clinically applicable methods used are the administration of exogenous progestins and prolonging luteal activity.

The synthetic progesterone analog altrenogest, administered orally, is the current pinnacle method of estrus suppression. Available as a biologically active oral formulation, the use of altrenogest provides the mare with an exogenous form of progesterone which simulates the presence of a functional CL and masks the effects of

estrogen produced by follicular activity. Altrenogest can be administered at any point in the estrous cycle, but requires daily oral treatment with 0.044 milligrams per kilogram (mg/kg) and administration for the duration of the desired effect [4]. In addition to the cost of this technique, altrenogest also carries safety concerns for those handling the product [5,6]. Injectable formulations of compounded long-acting progesterone and progesterone analogs have been evaluated as an alternative to oral altrenogest. However, the lack of regulation, variability in potency, questionable stability, and potential for injection site reactions create variable outcomes. Compounded formulations of altrenogest are also not approved by the United States Food and Drug Administration (FDA).

Various therapeutic techniques to prolong CL function for estrus suppression have been described. The use of an intrauterine glass ball has previously been regarded as the most common method of prolonging CL function [7]. This method mimics the presence of a conceptus and blocks the mechanism of luteolysis, which subsequently maintains the luteal tissue in the ovary. Intrauterine glass balls must be inserted and removed by a veterinarian. This method of estrus suppression is about 35% effective [8]. Beyond the low rates of success, the use of intrauterine glass balls can also have deleterious effects on the mare reproductive tract. The accidental placement of more than one glass ball can cause glass to fragment inside the mare's uterus [7]. As an alternative, the use of oxytocin as a method of prolonging CL function has gained much popularity as a method of estrus suppression [9].

Two protocols have been described for the use of oxytocin as a method of estrus

suppression. With knowledge of the date of ovulation (Day 0), 60 international units (IU) of aqueous oxytocin can be administered once daily intramuscularly (IM) from 7 to 14 days post-ovulation [10]. If the date of ovulation is unknown, 60 IU of aqueous oxytocin can be administered IM once daily for 29 consecutive days [11]. Multiple studies have shown that the use of oxytocin to prolong CL function is ≥ 70% effective. Although there is an increase in efficacy in comparison to the use of intrauterine glass balls, the need for daily treatment is a drawback to its clinical application.

To continue refining a more easily applicable oxytocin protocol for prolonging CL function, proprietary oxytocin formulation and improved protocols have been studied. In a preliminary study, three different proprietary formulations of slow-release oxytocin (SR-OT) were tested in an effort to eliminate the need for daily treatment [12]. Of the three formulations tested, one appeared to be more efficacious than the others, but required the need for further evaluation in comparison to a control group. To further evaluate the efficacy of a two-dose protocol, in Experiment 1, we hypothesized that administration of 2,400 IU of SR-OT IM once on day 7 and once again on day 10 post-ovulation would be an effective method of prolonging CL function in comparison to a non-treated control group. Consisting of only two treatments, this protocol would effectively reduce the number of required injections by 75% (8 treatments vs. 2 treatments) to achieve the same outcome as previously described oxytocin induced methods of estrus suppression.

Following the evaluation of a proprietary SR-OT formulation in Experiment 1, the return of the luteolytic mechanism during the period of prolonged CL function was

assessed in Experiment 2. During the normal luteolytic process, prostaglandin F2α (PGF2α) is the hormone responsible for luteolysis. While it is understood that oxytocin treatment for prolonging CL function disrupts cyclooxygenase-2 (COX-2) expression, it is not known when the luteolytic pathway returns after establishment of this prolonged state [13,14]. The average length of prolonged CL function is about two months [15]. In this study, the return of the luteolytic mechanism was evaluated by measuring an oxytocin stimulated pulse of 13,14-dihydro-15-keto PGF2α (PGFM), the main PGF2α metabolite. It was hypothesized that there would be a return of the luteolytic pathway in mares in a state of prolonged luteal activity. It was also the objective of this secondary study to determine when the luteolytic pathway returned in mares that were therapeutically treated with oxytocin for the purposes of estrus suppression. This research is highly relevant to the equine industry in order to develop additional understanding of the return of the luteolytic mechanism. With advanced understanding of this process treatments to further extend prolonged CL function for the purposes of estrus suppression in mares may be discovered.

CHAPTER II

REVIEW OF LITERATURE

The equine performance industry greatly values and has a high level of expectation for its animal athletes. Mares are no exception to this standard. Veterinarians are often approached by clients with the concern of how a mare's estrous cycle may be affecting her level of performance. For this reason, estrus suppression is a common practice in mares in all aspects of the industry with an emphasis on top athletes or prospects. Although there are many different estrus suppression techniques available, they come with mixed efficacy and unique drawbacks to each application. This review will investigate the intricate nature and manipulation of mare reproductive physiology, and different methods of estrus suppression. The use of oxytocin treatment to prolong CL function as an emerging and developing method of estrus suppression, and the return of the luteolytic mechanism during the period of prolonged CL function will also be discussed.

1. Estrous Behavior and Performance

Behavior conducive to breeding (heat) can be of great benefit in mares that have found roles in breeding programs. Estrous behavior in the mare is characterized by increased sexual interest in the stallion [16]. Mares can be "teased" in front of a stallion to determine the stage of the estrous cycle the mare is in. If the mare displays behavior that is indicative of heat, it can be assumed that the mare has a mature follicle approaching the point of ovulation and that there is no functional luteal tissue inhibiting

the estrogenic behavioral effects. Mares in heat may exhibit behavior including; wide-based hind limbs, lowered pelvis, raising the tail up and holding to the side, eversion of the clitoris (clitoral winking), and frequent voidance of urine. During this follicular phase of the estrous cycle the behavior of mares may range from mild interest in the stallion to a full display of reproductive signals and acceptance of the stallion mounting and copulation [17]. In contrast, mares in the luteal phase of the estrous cycle generally act agitated by the presence of a stallion and display aggressive behaviors such as switching their tail, pinning their ears back, tensing facial muscles, and biting or kicking at the stallion. When elevated, progesterone dominates over the behavioral effects of estrogen [18].

Behaviors associated with estrus have been shown to have a profoundly negative effect on training and performance [1]. Equine veterinarians often receive requests to manage the estrous cycle in performance mares because of difficulty in training and performance [19]. Clinical signs that have been associated with performance trouble include general difficulty in training, temperament changes, squealing, excessive urination, kicking, decline in performance, and abdominal discomfort associated with ovulation [2,20]. In performance settings, it is expected that the mare will exhibit a very high level of skill and the horse's disposition is a very important element in its ability to perform [21].

The first step in considering behavioral management of a performance mare is to determine if performance problems are truly related to the estrous cycle. If a mare is exhibiting inconsistency in training or performance, an evaluation by a veterinarian may

be necessary to rule out an injury or ailment as the cause of the behavior. When the mare is deemed to be healthy and sound, the next step is to determine what phase of the estrous cycle the mare is in. The mare should be observed in training during both estrus and diestrus to determine if estrus suppression is an appropriate treatment option. This sort of systematic evaluation can confirm what phase of the estrous cycle the behaviors in question are associated with. Using a stallion to tease a mare may be a necessary tool. The use of transrectal palpation and ultrasonography, and evaluation of serum progesterone concentrations can provide information necessary for a veterinarian to determine if follicular activity is associated with the exhibited performance issues. The most common issue in performance mares are behavioral changes associated with the estrous cycle [2]. When a veterinarian has confirmed association of performance issues with estrus, different estrus suppression methods can be implemented as discussed later in this review.

2. Introduction of Key Hormones

Reproductive cyclicity involves many different hormones. The interaction of the hypothalamus, the pituitary gland, and the ovaries of the mare is referred to as the hypothalamic-pituitary-ovarian (HPO) axis. This intricate network of endocrine tissue is responsible for most of the key hormones involved in the mare estrous cycle.

Progesterone is a steroid hormone that is primarily produced during diestrus and during pregnancy. Synthesized by the luteal cells of the CL and by the placental unit, progesterone is aptly named for its requirement during gestation in mares (and most other mammals) [22]. In a diestrus mare, and in early pregnancy, the CL is the main source of

progesterone [23]. The placental unit is the main source of progesterone and progestins from mid-gestation until term in the pregnant mare [23]. It is classified as a steroid hormone as it is derived from cholesterol. The target tissues of progesterone are the endometrium and myometrium. This hormone affects the tubular genitalia as well as physical and behavioral sex characteristics [23]. Progesterone also interacts with the anterior pituitary gland in a feedback system controlling luteinizing hormone. Progesterone is commonly regarded as the primary hormone in the maintenance of pregnancy [24]. It also inhibits the behavioral effects of estrogen, making this hormone a key player in most estrus suppression therapies [3,18]. Estrogen and progesterone have a dynamic relationship with antagonistic and synergistic effects. During diestrus, progesterone influences the downregulation of estrogen receptors. During estrus, estrogen stimulates the synthesis of progesterone receptors [25]. Of these two hormones that comprise the main steroid hormones of the mare estrous cycle, progesterone is the most dominant [26].

Estrogen is derived from the granulosa cells of the follicle, as well as the placenta, and the embryo. This hormone targets the hypothalamus and anterior pituitary gland and has influence on the reproductive tract and mammary gland of the mare [24]. The levels of estrogen directly affect sexual behavior of the mare [18]. Estrogen and progesterone have counteractive effects on the reproductive tract and on behavior [18]. Uterine tone is directly affected by the concentrations of progestins and estrogens [27]. The maximal uterine tone is reached during days 16 to 25 of pregnancy when progesterone being secreted from the CL is continued and the embryonic vesicle is supplying estrogens [27].

Estrogen and progesterone share many common synthetic factors. Both hormones stem from cholesterol derived pregnenolone, but there are two pathways proposed for the synthesis of estradiol-17β [23]. One pathway suggests that estradiol-17β is derived from progesterone through 17α-hydroxyprogesterone, a second pathway proposes an alternate route involving 17α-hydroxypregnenolone and dehydroepiandrosterone.

Prostaglandins serve many roles in equine physiology. All prostaglandins are derived from the cyclooxygenase pathway which converts arachidonic acid (**AA**) into the endoperoxides. These endoperoxides are then converted into various forms of prostaglandins. In general, prostaglandins function to control blood flow and activity of smooth muscle [23]. PGF2α is regarded as primarily responsible for luteolysis in the mare [28]. This hormone is derived from the uterine endometrium and promotes uterine tone and contraction by targeting the CL as well as the myometrium [24]. PGF2α targets the CL to cause luteolysis in late diestrus by acting through systemic circulation [23]. Unlike ruminants, the vasculature of the mare reproductive tract does not provide a local pathway for delivery of PGF2α to the ovary [29]. Instead, systemic circulation is required for PGF2α to target the CL and cause luteolysis. Systemic circulation targeting the CL can be utilized pharmacologically to manipulate the length of diestrus. Both native and synthetic forms of PGF2α are commercially available and can be used to terminate a CL at a desired time point [23].

Oxytocin is a neuropeptide synthesized in the hypothalamus and stored in the posterior lobe of the pituitary gland. Unlike most other mammals, oxytocin is not synthesized by the CL in mares [23]. Oxytocin primarily targets the endometrium and

myometrium in non-lactating mares. At the level of the endometrium, endogenous oxytocin stimulates synthesis and secretion of PGF2α. At the myometrium, oxytocin stimulates uterine contractions and motility [23]. In lactating mares, oxytocin also targets the myoepithelial cells of the mammary gland to stimulate the release of milk [24]. Therapeutically, oxytocin has many functions, some of which are contradicting. Exogenous oxytocin treatment can be used to enhance uterine clearance post-breeding, treat post-partum mares with a retained placenta, stimulate milk let-down, rarely aid in the timing of parturition, and also serve in luteolytic and anti-luteolytic functions [30,31]. This review will discuss in detail the ability of oxytocin to both stimulate PGF2α secretion from the endometrium, as well as its ability to inhibit the synthesis of PGF2α.

Gonadotropin releasing hormone (GnRH) is a neuropeptide hormone that is synthesized at the surge center of the hypothalamus. Every vertebrate species has the GnRH gene in its genome [23]. By way of the hypothalamic-pituitary portal system GnRH targets the gonadotroph cells of the anterior pituitary gland and initiates a cascade of hormonal events [23]. During the breeding season, elevated levels of GnRH stimulate the release of the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), in higher levels than during the non-breeding season [23,24]. The pituitary gonadotropins serve the same purpose among all domestic animals [23]. LH and FSH are biochemically classified as glycoproteins and are considered to be the gonadotropins involved in the mare estrous cycle. Both of these hormones are synthesized at the anterior lobe of the pituitary gland by the gonadotroph cells. FSH targets the granulosa cells of the ovary to stimulate follicular development and estradiol

synthesis. LH targets the theca interna and luteal cells of the ovary [24]. LH plays an important role in the process of ovulation and the formation of the CL which in turn produces progesterone. The chorion of the placenta also produces equine chorionic gonadotropin (eCG) in the pregnant mare. This hormone is synthesized by endometrial cups and acts in a luteotrophic fashion [23].

The mare estrous cycle has many physiologic components. The hormones of the HPO axis influence every aspect of reproductive behavior, conception, and seasonality.

3. The Mare Estrous Cycle

3.1 Seasonality of the Mare Estrous Cycle

The mare is seasonally polyestrous with regular follicular activity corresponding to duration of day length. The normal physiologic breeding season for mares is prompted by longer days and shorter nights, which in the northern hemisphere is typically late spring until fall (April through October) [32]. The increase in day length directly correlates to the number of mares having normal cyclic estrous activity [23]. This timing naturally allows for mating at the time of year that subsequently provides a newborn foal with optimal environmental conditions and nutrition for survival [23].

The physiologic breeding season is flanked on both sides by transitional periods of inconsistent follicular activity [33]. These phases are composed of irregular estrus behavior as the mare transitions from a winter anovulatory state into the photoperiod-induced breeding season and back into winter anestrous. Often, the transition into the winter anovulatory period is marked by a state of prolonged CL activity [34-36]. This phenomenon of long day breeding is regulated by a chain of hormonal and environmental

interaction that influence the ovaries (Fig. 1).

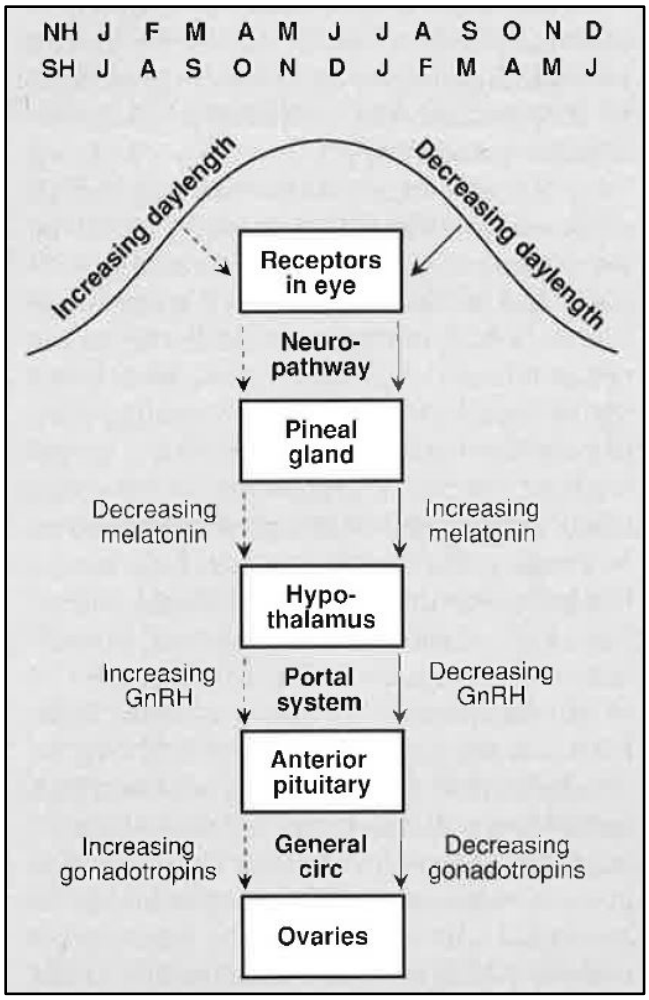

Fig 1. Mechanism for seasonality of the mare estrous cycle (Ginther, 1992)

The amount of light provided from daylength or artificial light sources directly stimulates photoreceptors in the eye. The photoreceptors then initiate a neuropathway effecting pineal secretion of melatonin. During shorter days of the year there is more melatonin secreted by the pineal gland than in longer days of the year. Melatonin secretion has an inverse relationship with GnRH secretion. Increased daylength decreases melatonin secretion and allows for increased GnRH leading to production and release of

LH and FSH during the breeding season. Systemic circulation delivers gonadotropins to the ovaries of the mare (Fig. 2). Of course, some mares exhibit behaviors and follicular activity that are inconsistent with the influence of daylength. Up to 35% of mares may exhibit unseasonable estrous associated with inconsistent follicular activity and some mares (10-15%) may continue their estrous cycle through the winter [37].

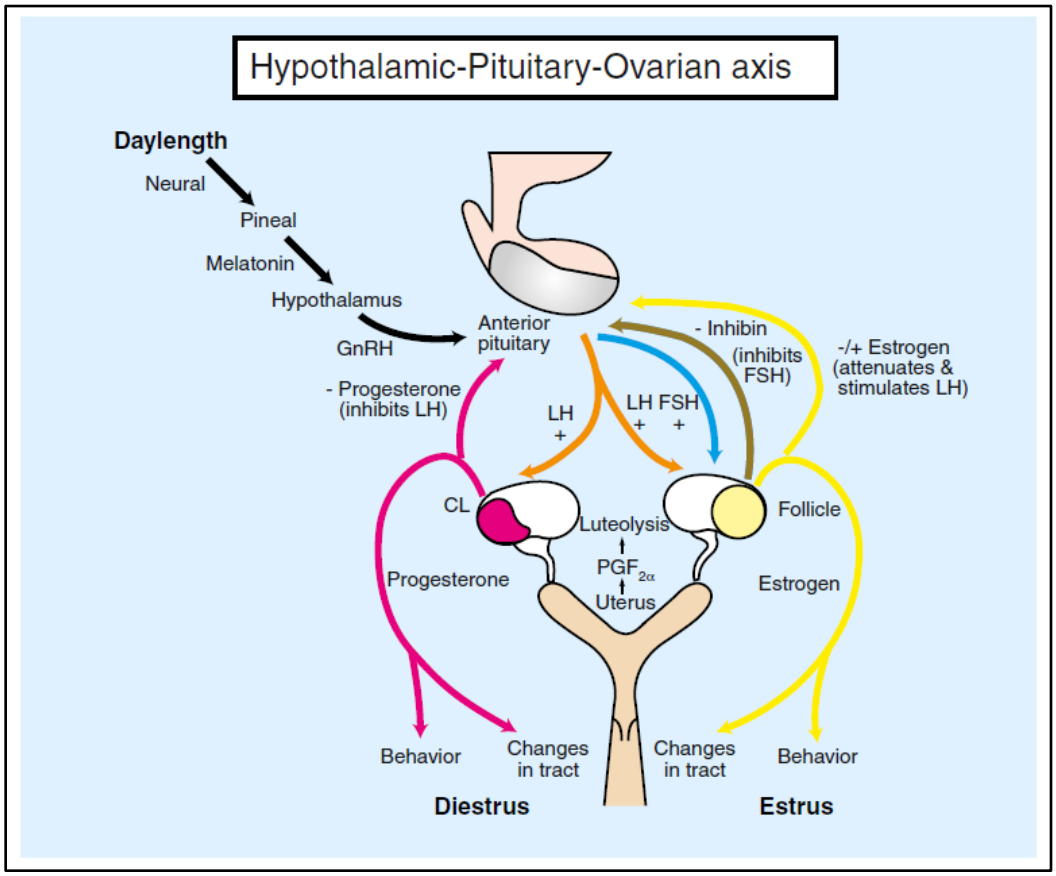

Fig. 2. Feedback mechanisms and seasonal regulation of reproductive organs involved in the HPO axis (Samper, 2009).

3.2 Advancing the Onset of the Ovulatory Season via Artificial Lighting

Understanding the effects of daylight on reproductive seasonality has provided advantageous tools to broodmare managers. With growing pressure to produce foals with

birthdays closer to the first of January, the artificial manipulation of daylight allows for advancement of regular follicular activity in the mare [38]. A total of 16 hours of light is sufficient to hasten the onset of the ovulatory season [39]. Artificial lighting should be initiated approximately 60 days before the desired beginning of ovulatory activity. The most preferred method of artificial lighting is to provide supplemental light at the end of each day to reach a total of 16 hours of light [38]. In this method, if sunrise was 8:00 AM and sunset was 6:00 PM, a source of artificial light would be illuminated from just before sunset until 12:00 AM (midnight) to reach the total prescribed exposure of light.

3.3 The Mare Estrous Cycle

There are two phases of the mare estrous cycle, estrus and diestrus, which are characterized by anatomical structures correlated with behavioral receptivity to the stallion. Estrus, diestrus, and the cycle in total, are determined to have an average length of 6.5, 14.9, and 21.7 days, respectively [23]. The estrus phase is reported to have the most deviation in length and may range from 3 to 12 days, but most often falls within five to seven days [19]. During the estrus phase, multiple follicles are present on the ovary with one or two dominant follicles as the main structures present on the ovary. These follicles produce estrogen, which promotes behaviors facilitating copulation with the stallion in the absence of high circulating concentrations of progesterone. The estrus phase is physiologically characterized by the growth and ovulation of a mature follicle during the absence of progesterone. The follicle, being a structure of transient nature, ovulates and transitions to become the CL. During the diestrus phase, which lasts about two weeks, the CL is the dominant structure present on the ovary. The CL produces

progesterone which promotes an endometrial environment suitable to maintain pregnancy. Progesterone also inhibits the behavioral effects of estrogen [18]. As a result, the mare is unwelcoming of the stallion, and sometimes even hostile towards him. The estrous cycle resets as the CL undergoes luteolysis in late diestrus of the non-pregnant mare (approximately day 14) and a new dominant follicle matures.

3.4 Anatomy of the Mare Reproductive Tract

The mare reproductive tract is a unique system that differs in many ways from other common farm species. The reproductive tract consists of the two ovaries, the tubular genitalia (oviducts, uterus, cervix, vagina, vestibule, and vulva), as well as the mammary glands (Fig. 3).

The ovaries are the master control center of the mare reproductive tract [23]. The ovaries of the mare are slightly larger than those of other common farm animals with a length of 4-8 centimeters (cm), width of 3-6 cm, and height of 3-5 cm. Individual mature follicles, the CL, and the ovary as a whole compared by body weight are larger than other farm animal species as well [23]. The ovary is kidney bean shaped with a concave surface known as the ovulation fossa on the ventral edge (Fig. 4). The ovary of a young filly is oval shaped, but a discernable ovulation fossa is formed by five months of age and the kidney bean shape is apparent by seven months of age [40,41]. The ovary positioning within the body cavity can be inconsistent as they may be moved by the intestines. The right ovary is on average 15cm caudal to the right kidney, as the left ovary is in closer proximity to the left kidney because that kidney has a more caudal positioning itself [42]. The ovary may be immediately adjacent to the uterine horn or as far away as five cm

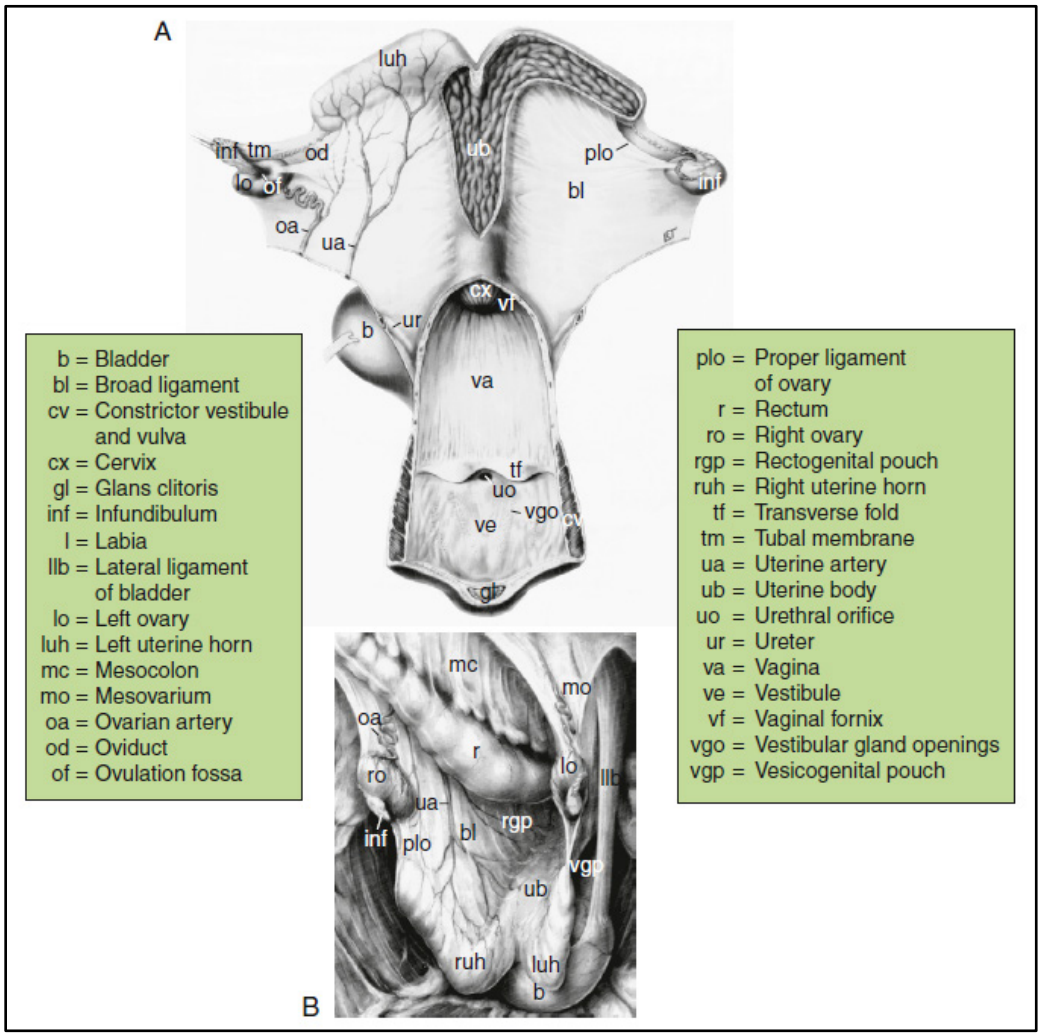

Fig. 3. The equine reproductive tract (A: Dorsal view, B: Lateral view) (Ginther, 1992).

estrogens. A mature follicle will ovulate to release the oocyte for fertilization and then [23]. In opposite fashion of most other farm species, the medullary region of the mare ovary is superficial. This medullary region of the ovary is the vascular zone of the ovary. The cortical region of the ovary lies deeper in the organ and only appears at the surface of the ovulation fossa. Unique to equine species, the ovulation fossa is the only point at which ovulation ensues [23]. As ovulation delivers an oocyte into the tubular reproductive tract, the follicle serves both gametogenic and endocrine properties. The

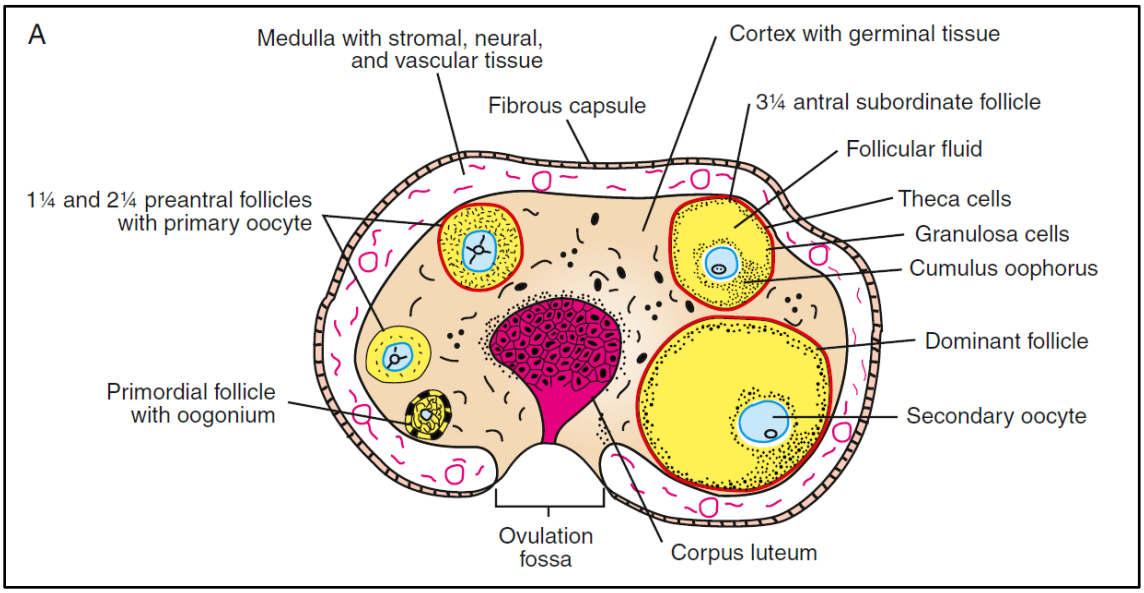

Fig. 4. Representative drawing of the morphological characteristics of a mare ovary (Samper, 2009).

function of the follicle is to house and develop the oocyte while actively producing transition into a CL. The CL serves only in an endocrine fashion as it produces progesterone. The follicle and CL operate as transitional endocrine glands controlling the environment of the tubular genitalia and behavior of the mare.

4. Follicular Dynamics

The underlying purpose of the mare estrous cycle is to develop and mature a follicle with the intent of ovulation and delivery of the maternal oocyte to the oviduct. These follicular dynamics begin at a histological level in the form of primordial follicles, long before visualization by transrectal ultrasound is possible. The primordial follicle is defined as the point at which an oocyte is arrested at a meiotic state and is enveloped in a layer of squamous cells [23]. This stage of follicular development is reached not long after a filly is born. As the primordial cell transitions into a new structure called the

primary follicle, the squamous cells are converted into cuboidal cells which eventually become the granulosa layer [23]. The transition from primordial to primary follicle is regulated in the mare by unknown mechanisms. In other mammals, it takes months for a primordial follicle to develop into a fully mature follicle with development of an antrum. An accurate timeline of folliculogenesis including this primordial-to-primary stage has not been determined in the mare. The number of primordial follicles that transition together is lower in mares than in cows. Mares may have about 100 follicles developing while cows develop 300 [23]. The mare, as with most other species, has not been reported to have mitotic development of oogonia. This presumably determines that there is a limited number of primordial follicles stored in the ovaries of each mare which cannot be replenished.

As primary follicles continue to develop, a great portion of small follicles grow and then undergo atresia. Atresia is the regression of these non-dominant follicles. Although the dynamics of small follicles are not well documented in mares, it is assumed that these follicles continually grow and become atretic through every cycle and reproductive state (estrus, diestrus, anestrous, pregnancy, pseudopregnancy, and postpartum) [43]. The group of follicles that grow simultaneously are referred to as a "major follicular wave." This follicular wave consists of five to ten follicles which grow at a rate of 2 to 4 mm per day [38]. The follicles within a wave typically begin growing synchronously, but eventually disassociate from one another during the selection process as one or two follicles continue to develop towards maturity and the others of the group undergo atresia. This differentiation in follicle outcome occurs when the largest follicle

reaches 20 to 25 millimeters (mm) [38].

The follicle that is most favored (grows the fastest) is referred to as the dominant follicle. This physiologically selected follicle grows to a large diameter (>30 mm). The mare, being a monovulatory species, will (in most cases) only develop one follicle that reaches maturity [23,44]. The dominant follicle will either ovulate, or it will regress (anovulatory wave). Secondary waves are also possible in the mare, in which, a second wave of growing follicles may ovulate a second dominant follicle during a state of diestrus [23]. Diestrus ovulations are more common in Thoroughbreds than in Quarter Horses or ponies [45].

Most often, the major follicular wave occurs in the form of a primary wave during late diestrus. This wave of follicles gives rise to the ovulation occurring during the follicular phase (estrus) of the estrous cycle [23]. The dominant follicle of the primary wave is carrying an oocyte that can potentially be fertilized. The primary wave is detectable by transrectal ultrasonography and palpation at about mid diestrus [43,46]. During the selection process, the dominant follicle will disassociate from the cohort of smaller follicles about seven days before subsequent ovulation. The disassociation is recognizable by the difference in growth rate (diameter) of the follicles.

The mare estrous cycle only consists of one or two follicular waves. The interovulatory interval (IOI) is determined as the number of days from one ovulation until the next. In estrous cycles with two follicular waves, there is one primary follicular wave producing a potentially fertilizable oocyte, and an earlier secondary wave, which may produce a large anovulatory follicle, or a diestrus or secondary ovulation [47]. Breed also

has influence on the follicular activity of the mare. Quarter Horses and pony breeds have lower levels of follicular activity than that of Thoroughbred and Standardbred mares [46,48].

5. Hormonal Aspects of Follicular Dynamics

Follicular waves start with about 100 primordial follicles, but end with a single dominant follicle reaching ovulation [23]. The dominant follicle will subsequently become the CL after ovulation. Slightly before the time of ovulation, a gradual increase in hypothalamic GnRH results in elevated FSH for the duration of diestrus. This influences the developing follicles to synchronously grow into small antral follicles. As the follicles in the primary wave increase in size, they also increase in the production of inhibin from the follicular granulosa cells. Inhibin works as a negative feedback for FSH. This negative feedback decreases the amount of FSH gradually from about 13 days after ovulation (Fig. 5).

The decrease of FSH and increase of LH and inhibin continues throughout the point of luteolysis. At about day 14, PGF2α from the endometrium systemically circulates in the mare targeting the CL. PGF2α originates from the non-gravid uterus of the mare and is stimulated by oxytocin in a pulsatile manner [49,50]. During this time the follicles are still growing at a generally synchronous rate, but the number of recruited follicles has dropped to about 10 or less because of unknown mechanisms. By day 17, the CL has regressed significantly in size and function. Without the influence of progesterone, the follicles continue to grow and the dominant follicle is selected.

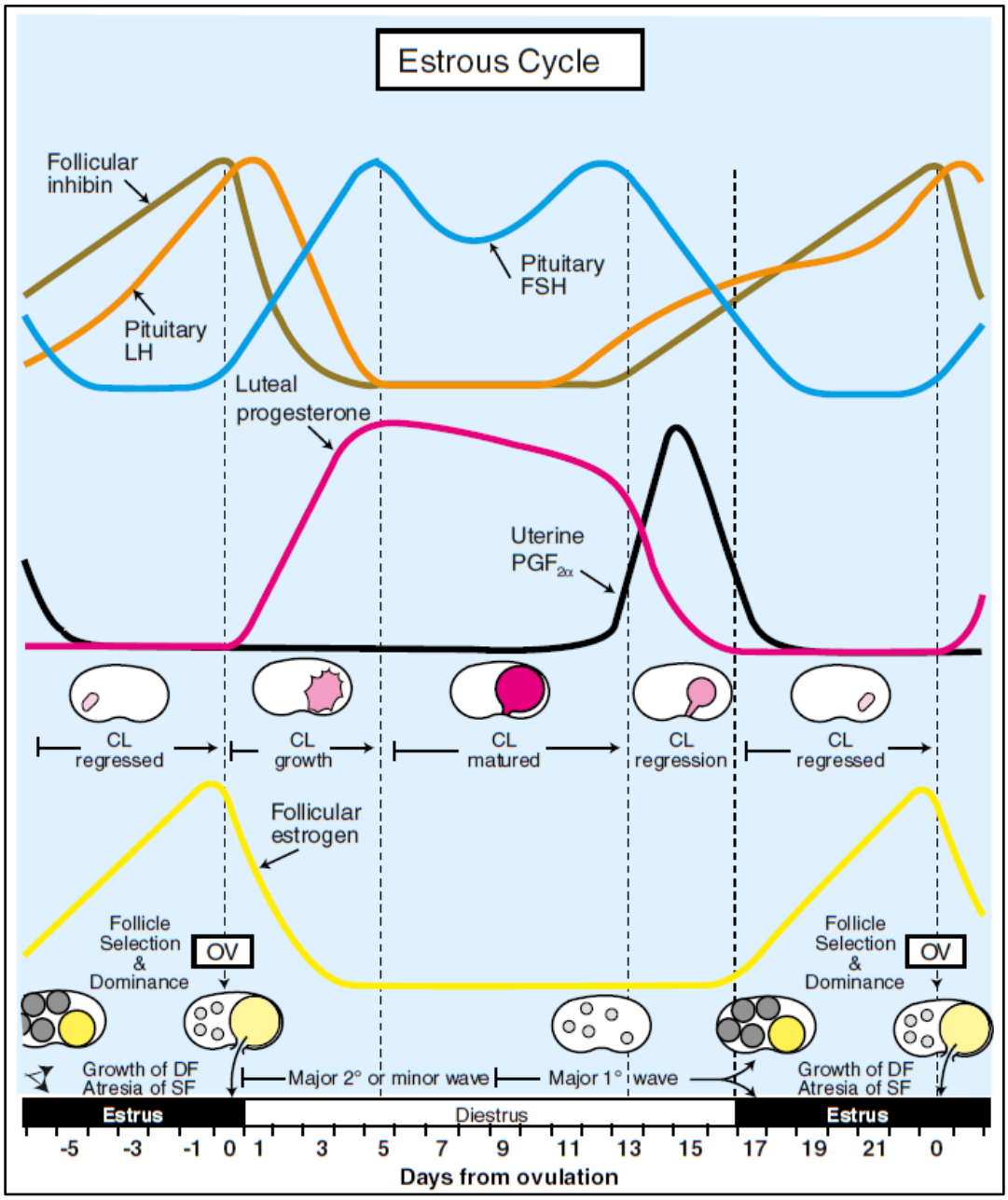

Fig. 5. Profiles of hormones associated with the equine estrous cycle (Samper, 2009).

Follicular estrogen increases and the mare becomes more receptive to mating with the stallion. After the dominant follicle is selected it continues to mature and develop, producing inhibin and estrogen, as the subordinate follicles undergo atresia. The dominant follicle will typically grow to 35 mm or larger before ovulation. Follicular

estrogen typically peaks just before ovulation and has a synergistic effect with LH resulting in low levels of FSH [38]. The dominant follicle often becomes irregular or guitar pick shaped just before ovulation. This is because of the follicle's conformation to the ovulation fossa of the ovary. Ovulation is stimulated by a surge of LH [51]. The rupture and release of the oocyte by the dominant follicle characterizes ovulation and marks the end of one IOI and the beginning of the next.

After ovulation, the once dominant follicle begins to transform by undergoing luteinization to create the CL. Unlike the CL in ruminants which has a delay in the production of progesterone after ovulation, in the mare, the CL begins producing progesterone immediately after ovulation [23,52]. Within 24 to 48 hours, progesterone counteracts the behavioral effects of estrogen making the mare antagonistic towards mating with the stallion [18]. LH and inhibin steeply decrease after ovulation as FSH increases and remains high during the luteal phase of the estrous cycle. Small pulses of LH continue for a short period of time after ovulation to aid in the development of the CL.

6. Luteolysis

The luteal phase of the estrous cycle in mares is characterized by the presence of a CL and the secretion of progesterone. This hormone influences behavior as well as the tubular genitalia of the mare. Specifically, progesterone prepares the reproductive tract to receive an embryonic vesicle and begin early gestation. If an embryonic vesicle is recognized within the uterus, complex mechanisms block the occurrence of luteolysis. With the absence of an embryonic vesicle, the uterus maintains the ability to reset and

initiate a new estrous cycle.

As PGF2α is secreted in pulses over a 23-hour period, the CL goes from being the controller of reproductive cyclicity in the mare, to a remnant of the past [53]. Luteolysis results in the loss of function of the CL as a consequence of targeting by PGF2α [54]. At about 14 days after ovulation, a non-gravid uterus signals that it is ready to enter another follicular phase. In order to lyse the CL, the uterus secretes PGF2α. This luteolytic hormone is secreted in small pulses before luteolysis, but during the time of luteolysis the concentration of PGF2α is about six-times higher than before [55]. This transition from a pre-luteolytic to a luteolytic stage only takes about one hour [55]. As marked by progesterone concentrations, the events of luteolysis begin on day 14 and are concluded around day 17 when progesterone falls below 1.0 nanogram per milliliter (**ng/mL**) [38]. A complete understanding of luteolysis in the mare has yet to be established and is a reason that our lab has endeavored to further characterize this process.

6.1 The Role of the Uterus in Luteolysis

The relationship between the uterus and the CL is very important in the mare estrous cycle. Uterine mechanisms are responsible for the maintenance or destruction of the CL [56]. In the non-pregnant mare, the uterus is responsible for the secretion of PGF2α. In the pregnant mare, the uterus undergoes specific changes which disrupt the processes of luteolysis [57].

In the non-pregnant mare, luteolysis occurs as a result of PGF2α secretion and systemic delivery to the CL [58]. The regression of the CL as a result of pulsatile PGF2α secretion marks the end of diestrus [59]. The vasculature of the mare reproductive system

dictates that uterine derived PGF2α must travel systemically to reach the CL [29]. This delivery is different than most other farm animal species which exploit localized pathways of vasculature [60]. The differences in luteolytic pathways between ruminants and horses have been studied extensively by Ginther et al. to uncover how the vasculature (local vs. systemic) affects the route of PGF2α [61]. The close proximity of the utero-ovarian vein and the ovarian artery in ruminants allows for a local transfer of PGF2α from the uterus to the ovary. It is assumed that the intimate relationship of anti-directional blood vessels allows for the ability of the animal to exploit an amount of counter-current exchange. This transfer is possible because PGF2α is fat soluble and can travel along lymphatic and venous vessels to make its way to the ovary [62]. Because of the local nature of luteolysis in ruminants, there is also an ipsilateral association between the uterus and the CL in the recognition of pregnancy [63].

In the mare, the vasculature of the uterus and ovary do not share the same intimate relationship that is seen in cattle and secretion of PGF2α targeting the CL occurs systemically [29]. The systemic circulation targeting the ovary has been demonstrated in many studies. Using three different routes of administration (local: intrauterine or intraluteal; systemic: IM) and three different doses of PGF2α (0, 0.25, or 1.25 mg) there was no significant difference in the route of administration on the occurrence of luteolysis in mares [64]. The actions of systemic circulation and delivery of uterine secretions are further supported by embryonic characteristics of the maternal recognition of pregnancy in the mare [63]. Embryonic mobility in the mare has no ipsilateral or unilateral relationship between the uterus and ovary (discussed in detail later in this

review) [65].

The half-life of PGF2α is very short, making this hormone very hard to measure directly in the systemic circulation. The main metabolite of PGF2α in horses and cattle is 13,14-dihydro-15-keto PGF2α (PGFM) [66,67]. Because the half-life of PGFM is longer than that of PGF2α, the concentration of circulating metabolite is often measured in order to represent concentrations of PGF2α. In comparisons of progesterone concentrations in response to simulated PGFM pulses, it is apparent that complete luteolysis requires multiple pulses of PGF2α [68]. During the preluteolytic window, rhythmic PGFM pulses were not detected, but rhythmic pulses of PGFM were indicated in seven of nine mares during luteolysis and post-luteolysis in a study by Ginther et al. [55]. The requirement of sequential pulses of PGF2α is also characteristic of luteolysis in cattle [69]. Following luteolysis, the remaining remnant of the CL is referred to as the corpus albicans (**CA**) [70].

6.2 The Role of Oxytocin in Luteolysis

The uterine preparation for luteolysis begins long before the luteolytic window. Although our knowledge of the luteolytic mechanism is improving, there is room to further characterize the event of luteolysis. Oxytocin has been described in both horses and ruminants as an important factor in the chain of the luteolytic process [50,61]. In ruminants, it is known that oxytocin from the posterior pituitary gland targets its receptors at the uterine endometrium [61]. The interaction of estrogens with the endometrium during late diestrus act as a proliferative stimulant of oxytocin receptors in ruminants [71,72]. It has been shown that the presence of steroid hormones is critical for

the development of oxytocin receptors [71]. A positive feedback luteolytic mechanism involving oxytocin and PGF2α has been described for ruminants. Posterior pituitary derived oxytocin targets the endometrium stimulating the synthesis and secretion of PGF2α which targets the CL. In ruminants, the CL provides an additional source of oxytocin which continues to target the endometrium creating a secondary luteolytic pulse of PGF2α [73]. In horses, the interaction between oxytocin and uterine PGF2α are not understood as well.

In mares, the CL is not described as a source of oxytocin involved in luteolysis [74]. Oxytocin in mares is involved in luteolysis by way of a different positive feedback mechanism. The endometrium and the posterior pituitary gland have both been shown to be producers of oxytocin [49,50]. It is proposed that small amounts of uterine derived oxytocin interact with oxytocin receptors present on the endometrium in late diestrus and consequentially stimulate PGF2α secretion which targets the CL. The circulating PGF2α influences the secretion of oxytocin from the posterior pituitary which then targets the endometrium causing a dramatic luteolytic increase of PGF2α [75]. As mentioned previously, multiple pulses of PGF2α are necessary for complete physiological luteolysis [68].

The pathway of oxytocin develops some complexities at the endometrium where it stimulates PGF2α synthesis and secretion. As oxytocin targets its receptors at the endometrium, it stimulates the activation of phospholipase A2 (**PLA2**), which is critical in the release of arachidonic acid (**AA**) from the cell membranes [76,77]. AA is the substrate necessary for all prostaglandin synthesis through the cyclooxygenase pathway.

The cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) proteins convert AA into prostaglandin G2 (PGG2) and prostaglandin H2 (PGH2), which are known as endoperoxides. PGH2 is converted to PGF2α by mediation of prostaglandin F synthase (**PGFS**) [13]. The involvement of COX-2 in luteolysis and maternal recognition of pregnancy is greater than the involvement of COX-1. The oxytocin-prostaglandin pathway has been evaluated in various studies. It has been shown that oxytocin administered during late diestrus results in an immediate spike of PGFM [78]. It has also been determined that PGFM in response to oxytocin reaches a maximum around the window of luteolysis [79].

At the time of luteolysis in the mare, COX-2 is highly expressed in the endometrium. This parallels the expression of COX-2 in ewes and in cows during late diestrus [80,81]. Alternatively, COX-2 expression is significantly lower in the pregnant mare. It is assumed that the equine conceptus inhibits COX-2 and subsequently interferes with the synthesis of PGF2α and the luteolytic process [76].

The presence of oxytocin receptors at the endometrium also fluctuates depending on the phase of the estrous cycle of the mare [78]. Prior to day 10 after ovulation, the concentrations of oxytocin receptors and the expression of COX-2 are too low to create a sufficient luteolytic response [76]. After day 10 post-ovulation, the required enzymes and oxytocin receptors are expressed in high enough quantities to cause luteolysis. There is a direct correlation between the ability of the endometrium to secrete PGF2α and elevated concentrations of oxytocin receptors in late diestrus in normal cycling mares [82].

Although oxytocin is proposed as a key element in the processes of luteolysis, it

has also been realized as a clinically applicable method to inhibit the functions of luteolysis. These contradicting effects are achievable through a greater understanding of the hormonal aspects controlling luteolysis.

7. Maternal Recognition of Pregnancy in the Mare

The mare exhibits some unique features in the events leading to maternal recognition of pregnancy. The concept of embryonic mobility and the lack of understanding of the chemical signaling from the embryo may be the most puzzling, but crucial, of them all. After fertilization of the oocyte in the oviduct, the embryo undergoes oviductal transport. Beginning three to four days after fertilization, embryonic secretion of prostaglandin E2 (PGE2) stimulates transport of the embryo toward the utero-tubal junction [83]. PGE2 secretions from the embryo are significantly higher around day five to six and elevate to even greater concentrations following uterine entry around day six [83]. Continuous oviductal administration of PGE2 has been shown to accelerate the embryonic stage of oviductal transport [84]. Although the embryo secretes appreciable amounts of PGE2 and a pharmacologic dose of PGE2 stimulates prolonged CL function in non-pregnant mares, there is no conclusive evidence that PGE2 is responsible for maternal recognition of pregnancy in the mare [85,86].

After the embryo enters the uterus, it is able to be observed by transrectal ultrasound around nine or ten days after ovulation [87]. The mobility of the equine conceptus in the uterus is vital to the maternal recognition of pregnancy, and is a very distinguishable feature compared to other domestic species [88]. Embryonic mobility fluctuates from entry into the uterus until fixation, and can be visualized by the use of

transrectal ultrasonography. The amount of mobility peaks between days 11 and 14 during which time the embryo may be traversing across the endometrium more than 12 times a day [89]. This extensive travel of the embryo is a physical requirement for the maternal recognition of pregnancy. When embryo mobility was artificially restricted to one uterine horn; one uterine horn and the uterine body; or one horn plus the uterine body and 80% of the second horn; one of five, two of four, and four of four mares successfully maintained pregnancy, respectively [90]. The previous example indicates the necessity of the embryo to freely travel and interact with at least two-thirds of the endometrium. This study further demonstrated the necessity of progesterone to maintain pregnancy [90]. When the embryo in mares was restricted to one uterine horn and the mare was subsequently supplemented with progestins, pregnancy was maintained.

The requirement of embryonic mobility is clear, but the exact mechanisms by which the embryo inhibits luteolysis are not clear. COX-2, a key enzyme in the pathway of prostaglandin synthesis, is inhibited by the presence of the conceptus in the uterus [76]. It is hypothesized that the disruption of a preliminary step to COX-2 expression may be an underlying cause to its inhibition. There is also a disruption of oxytocin receptor expression in early pregnancy. Oxytocin receptor expression is similar in pregnant and non-pregnant mares on day 12, but is significantly lower in pregnant mares than in non-pregnant mares on day 14 post-ovulation [91].

In comparison to other common farm animal species, there is a lack of knowledge of conceptus derived chemical signaling for the purposes of maternal recognition of pregnancy. In pregnant ewes and cows, ovine and bovine-interferon tau play an essential

role in the prevention of luteolysis [92,93]. In swine, conceptuses secrete multiple interferons, but the role of those interferons are not known to directly affect pregnancy [93]. Conceptus derived estrogens inhibit luteolysis in swine by influencing upregulation of prolactin receptors and blocking prostaglandins from being delivered to the ovaries via uterine vasculature. Instead, prostaglandin is deviated towards the uterine lumen where it is metabolized to a form that will not cause luteolysis [94]. In mares, neither estrogen nor an interferon component have been identified as a significant chemical signal for the maternal recognition of pregnancy. In a 1994 study by Vanderwall et al., eleven non-pregnant mares were treated with continuous intrauterine infusion of 6 μg/day of estradiol-17β [85]. Although six of the eleven mares exhibited prolonged luteal function until day 30 post-ovulation, it was determined that treatment with estradiol-17β was not significantly different from sham-treated mares in which four of eleven mares exhibited prolonged luteal function ($P = 0.34$). There has not been any evidence of an interferon-type gene associated with the equine embryo [95].

The underlying mechanisms for the inhibition of the luteolytic pathway in pregnant mares are not completely understood. More research must be done on this intricate event as complexities of equine pregnancy are unique and comprise an extensive list.

8. General Goal of Estrus Suppression

Some mares exhibit unwanted behaviors that are not associated with injury or ailment but adversely impact performance. Mare owners may choose to evaluate behavior in correspondence with the mare's estrous cycle to determine if the undesirable behavior

is correlated with the estrus phase. During estrus, a mare may become difficult to train or ride, may exhibit sensitivity in her flanks, and show common signs of estrus behavior. If the mare's problematic behavior is carefully evaluated and determined to be directly associated with estrus, mare owners may choose to address this problem using estrus suppression techniques.

The goal of all estrus suppression techniques is to address the deleterious behaviors associated with the estrus phase of the cycle. By accomplishing this goal, performance mares can compete at their full potential without the distractions of the estrous cycle taking effect, mare owners can address estrous related behavior to work in a safer environment with their horses, and the behavior of a mare can become more consistent to aid in the detection of injury or illness.

The most common estrus suppression therapies can be grouped into two general methods. First is the administration of exogenous progesterone, which is most commonly performed with the use of oral altrenogest supplementation. The second method is the therapeutic maintenance of CL function. By preserving CL function, the mare will have a continued source of endogenous progesterone. The presence of progesterone serves to counteract the behavioral effects of estrogen which are high during estrus. In a typical 21 to 22-day estrous cycle, a mare may exhibit estrus behaviors for five to seven days, sometimes even longer [18].

Several techniques to suppress estrus behavior have been examined with varied success. The administration of oral exogenous progestins is currently the most prevalent method to suppress estrus, but this method also has associated risks and consequences.

The oil based altrenogest products are easily absorbed through the skin creating risk to human administrators. The FDA reported in 2018 that their agency had received over 130 reports of accidental human exposure to altrenogest products. Most of the reports regarding altrenogest indicated adverse effects in the menstrual cycles of women, and decreased libido in men [6]. Besides the risk to human health, most altrenogest products are expensive and require daily oral administration. In previous years, the use of an intrauterine marble was perceived to be the best method of prolonging CL function for estrus suppression, but this method has since been documented to have deleterious reproductive consequences [7]. As an alternative to intrauterine glass marbles and daily oral progestins, several therapies using oxytocin have been explored. Oxytocin therapies for estrus suppression inhibit the luteolytic mechanism causing luteal activity to be sustained.

There are additional methods of estrus suppression that involve immunological manipulation, surgery, or the establishment of pregnancy. Therefore, the following review will summarize the current body of research available on literature on all methods of estrus suppression in mares. Methods involving oxytocin are considered a hormonal method of sustaining endogenous progesterone and are described in a stand-alone section.

9. Prolonged Luteal Activity

The occurrence of prolonged CL function in mammals is quite a common phenomenon. Some of the terms that can be used to describe the continual function of a luteal gland are “prolonged luteal activity,” “persistent CL,” or “prolonged CL function.” The term “prolonged diestrus” does not imply a CL deriving from an ovulated follicle,

but rather suggests an extended period of diestrus behavior as the mare is not receptive to a stallion. In mares, the occurrence of this prolonged secretion of progesterone is often associated with pregnancy, but can also occur spontaneously in non-pregnant mares. The prevalence of idiopathic prolonged luteal activity in mares is about 10% and lasts for about two months [15]. Ginther describes prolonged luteal activity as the determination that life of a given luteal gland has been extended [96]. It is generally accepted that estrus suppression techniques which prolong luteal activity are successful if progesterone concentrations remain above 1.0 ng/mL for a minimum of 30 days. The presence of progesterone greater than 1.0 ng/mL indicates a functional CL present on the ovary of the mare, but there is a chance that progesterone may be produced by a secondary CL or a diestrus ovulation producing a subsequent CL. The occurrence of diestrus ovulations in the mare or secondary follicles undergoing luteotrophic transitions influences research. Most studies investigating prolonged CL function use transrectal ultrasonography and palpation in addition to serum progesterone evaluation.

10. Hormonal Methods of Estrus Suppression

10.1 Oral Progestins (Altrenogest)

The current gold standard for estrus suppression therapies available to practitioners is the oral progestin altrenogest which was approved for use in mares by the FDA in 1983. Although altrenogest is now available in FDA approved generic formulations, the most common label name is ReguMate® from Merck Animal Health (Intervet, Inc.). Oral altrenogest is recommended for use at a dosage of 0.044 mg/kg daily. Although there is a great benefit in altrenogest's ease of use, there are also

disadvantages to its use. Altrenogest is very absorbable through the skin and can be unsafe for administration by women of childbearing age, or pregnant women. This oral administration of altrenogest can also become very expensive and calls for daily treatment of the mare.

10.2 Injectable Progestins

Since the 1960s, progesterone in oil has been used to suppress the behavioral effects of estrous in mares [97]. The use of exogenous progesterone was accepted as a method of behavioral estrus suppression at a dose of 0.2 mg/kg. Although progesterone in oil was deemed to be effective, the requirement for daily administration and prevalence of injection site reactions were undesirable [19]. In an effort to improve the clinical application of injectable progesterone, a compounded version was created in a long-acting formulation of 1.5 grams (g). This formulation successfully maintained serum progesterone concentrations above 1.0 ng/mL for about 10 days, but did not eliminate the injection site reaction [98]. There are also compounded injectable altrenogest formulations, which are not FDA approved, but have been reported to block estrous behavior for 12 and 15 days in doses of 225 mg and 450 mg, respectively. In the study of an injectable 500 mg dose of altrenogest it was reported that estrous behavior was suppressed for approximately 30 days [99]. Although compounded long-acting progesterone and altrenogest are not approved by the FDA, compounded long-acting altrenogest is available in Australia by the name of Readyserve from Ceva Animal Health.

Medroxyprogesterone acetate (MPA) is an injectable long-acting progestin that

has been used in women as a human contraceptive (Depo-Provera, Pfizer). MPA has shown to be efficacious as a tool for wildlife contraception and is even used in some zoos for the uses of estrus suppression, contraception, or even management of pregnancy (St. Louis Zoo, St. Louis, MO). This therapy is also effective in queens and bitches, but comes with an array of undesirable side effects [100]. In mares however, this biological progestin does not have the same effect on the natural reproductive cycle. Although there are anecdotal reports of the use of MPA to address performance problems in mares, research shows that mares treated with MPA continue to display estrous behavior in a normal pattern [101]. Mares injected intramuscularly with 1,600 mg of compounded MPA on day 7 post-ovulation and repeated doses of 400 mg on days 14, 21, 28, 35, and 42 post-ovulation exhibited no deviation of estrous behavior in comparison to a saline-treated control group [101]. The treated group also showed no differences in follicular activity, progesterone or LH concentrations.

10.3 Off-Label Implants

Different types of implanted devices have been used in cattle for estrous cycle synchronization and for improvements in feedlot performance. These devices most often use progestins to influence the effects of behavioral estrus. Synovex-C ® (Fort Dodge Animal Health, Overland Park, KS) and Controlled Intravaginal Drug Release (CIDR) devices have been hypothesized to be efficacious for the purpose of estrus suppression in mares, but research shows that neither treatment is successful [2,19]. It is hypothesized that these implants do not work for the purposes of estrous suppression in mares because of the inability of great enough quantities of the hormone to be absorbed [102].

Other hormonal implants have been tested with varied success. Deslorelin acetate is one treatment that has mixed use and variation of efficacy. Most often, GnRH analogs, such as deslorelin, are used for their intended purposes to aid or hasten the ovulation of a follicle greater than 35 mm in diameter [103-105]. Deslorelin, in an implantable pellet form, is known as Ovuplant. In one study, most mares that were administered two 2.1 mg Ovuplant doses at one treatment exhibited suppressed follicular activity for more than 30 days [106]. It was hypothesized that the administration of three 2.1 mg Ovuplant doses at one treatment would influence the ovaries of the mare to become quiescent. Although the increased dose of the GnRH analogue did increase the IOI to 36.8 days, it did not manifest into arrest of ovarian function [107].

Nogestomet (Crestar, Intervet Australia, Bendigo East Victoria, Australia) and Levonorgestrel (Norplant, Wyeth, Collegeville, PA) have both been marketed as methods of estrus suppression in mares, but there is a shortage of refereed information available to determine the reliable efficacy and treatment protocol.

10.4 Glucocorticoids

Reports have shown successful suppression of estrus using glucocorticoids. In a 1982 study, by Asa and Ginther, 30 mg/day of dexamathosone was administered starting on day 10 post-ovulation. Treatment with this synthetic glucocorticoid successfully suppressed estrous behaviors and interfered with ovulation in seven of eight mares [108]. Although there is evidence of success using dexamethasone, a survey from 2007 reflects that using glucocorticoids was not a preferred option for veterinarians attempting to suppress estrus in mares [109].

11. Prolonging CL Function as a Method of Estrus Suppression

11.1 Induction of Late-Diestrus Ovulation

In contrast to most other species, mares can ovulate new follicles while being in a state of diestrus. This can occur as a spontaneous phenomenon or be influenced by an ovulation induction agent. Spontaneous mid or late diestrus ovulations may result in prolonged CL activity and maintain elevated progesterone through the point of luteolysis. This resistance to PGF2α is most likely because the CL deriving from the diestrus follicle is not mature enough to be susceptible to the luteolytic hormone [110]. In an evaluation of breed prevalence, this phenomenon of diestrus ovulation occurs in about 20% of Thoroughbreds and Quarter Horses during diestrus, yet is very unlikely in pony mares [45,111].

Using ovulation induction agents such as human chorionic gonadotropin (**hCG**), it is possible to induce large diestrus follicles (suggested follicles ≥ 35 mm in diameter) to ovulate even in the presence of high concentrations of progesterone. A 2006 study by Hedberg et al. indicated that the ovulation of diestrus follicles was a possible therapeutic tool for estrous suppression by prolonging luteal activity [112]. Five mares were evaluated during diestrus using transrectal palpation and ultrasonography for a diestrus follicle ≥ 30 mm. When an appropriate sized follicle was detected, 3000 IU of hCG was administered as to induce ovulation. Three of the five mares evaluated successfully exhibited prolonged luteal activity, one mare was treated with hCG but did not ovulate the diestrus follicle, and the fifth mare never developed a diestrus follicle large enough to administer the hCG treatment.

The induction of late-diestrus ovulation is a viable treatment option for estrus suppression, but is not an optimal choice for most horse owners. The need for repeated transrectal examinations and the chance that the mare may never develop a diestrus follicle large enough to treat leaves a chance that the owner will just be wasting time and money.

11.2 Intrauterine Marbles

Sterile glass marbles ranging from 25 mm to 35 mm placed into the uterine lumen have been used to suppress estrus in mares. These marbles are inserted following ovulation and are assumed to mimic the presence of a conceptus in the uterus. The previous understanding was that the marble impeded the ability of the endometrium to secrete PGF2α by its mobility throughout the uterus mimicking a conceptus or that the marble mildly damaged the endometrium impairing its ability to maintain the luteolytic mechanism. It has recently been shown that other types of intrauterine devices (IUD) in mares inhibit the upregulation of COX-2, which could also be an effect that intrauterine marbles may have on the endometrium. The use of a sterile glass marble for estrus suppression is past its prime as veterinarians have concluded that negative consequences outweigh the benefits [113]. The efficacy rate to prolong CL function (approximately 35%) using intrauterine marbles is fairly low compared to other estrus suppression therapies [8]. In some cases, the marble may pass back through the cervix and be expelled immediately, or could be expelled the next time the mare enters estrus and her cervix is relaxed. There have been reports of glass marbles shattering inside the mare's uterus. This is most often caused by placement of a second marble without the removal of

the initial marble. The use of intrauterine marbles also has other associated side effects that have been clinically documented including colic-like symptoms and pyometra [114,115].

11.3 Other Intrauterine Devices

Research on proprietary IUD methods of estrus suppression has been pursued as the need for an alternative to an intrauterine marble has become apparent. These proprietary units range in size and structure, as well as materials. In a study by Rivera Del Alamo et al., a 20 mm water filled polypropylene ball was deposited in the uterus of mares on day 3 post-ovulation. Thirteen of 15 treated mares exhibited prolonged luteal function and it was determined that the abundance of COX-2 was decreased in non-pregnant mares treated with the IUD [77]. More recently, a self-assembling IUD (Upod) has been analyzed. The Upod is a set of three polymer-coated elliptically shaped magnetic units which self-assemble into a "ring" conformation when deposited into the uterus of the mare. In the first experiment in which the Upod was deposited into the mare during estrus 13 of 15 mares exhibited prolonged luteal activity with the average length extending to 73.4 days [116]. A second experiment in the same report resulted in 12 of 14 mares exhibiting prolonged luteal activity for an average length of 51.3 days when the Upod was deposited in the mare at a random point of the estrous cycle. Although all mares in the study retained the Upod, there was an elevated level of endometritis. IUDs have many applications for estrus suppression and synchronization, but the potential for endometritis may create hesitation for horse owners considering this option.

11.4 Intrauterine Infusion of Plant Oils

In a 2011 study, Wilsher and Allen demonstrated that a single intrauterine infusion of 10 mg of slow-release estradiol in fractionated coconut oil was an effective method of prolonging CL function in mares [117]. With treatment administration on day 6, 8, 10, 12, and 14 post-ovulation prolonged CL function was observed in 25%, 75%, 92%, 83%, and 50% of mares, respectively. The intrauterine infusion of plant oils on day 10 post-ovulation showed similar results when either coconut oil or peanut oil was administered alone (92% for each), indicating that estradiol was not necessary to induce luteostasis in mares. There is a need for further research on the intrauterine infusion of plant oil to uncover if this technique modulates prostaglandin production or secretion. It is hypothesized that the assortment of fatty acids found in both coconut and peanut oils is the underlying factor contributing to prolonged CL function using these techniques.

12. Immunologic Methods of Estrus Suppression

GnRH stimulates the pituitary gland to release LH and FSH causing follicular growth and eventual ovulation. Removing this stimulus for gonadotropin secretion reduces or halts ovarian activity which stops estrogen secretion and ultimately nullifies estrous behavior. The commercial GnRH vaccine known as Equity (manufactured by Zoetis) is not currently available and has never been FDA approved. This vaccine acts to create antibodies against GnRH making it impossible for the endogenous GnRH to act on its receptors at the pituitary gland. Equity was previously available in Australia, but has since been removed from Zoetis' lineup of products. When treated with this vaccine, some mares still continued to display estrous behavior despite the lack of ovarian activity,

which, as noted previously, is referred to as paradoxical estrous behavior. Aged mares may require repeated vaccination for treatment to be efficacious. Most mares treated had extremely long periods of inactive ovarian function while some mares never regained any ovarian activity [118]. There is no reversal to a GnRH vaccination which is a major disadvantage to this method of estrus suppression.

13. Surgical Methods of Estrus Suppression

One of the most puzzling phenomena in mare reproduction revolves around the surgical removal of the ovaries. This remedy to resolve issues associated with estrus behavior is, in theory, a "cure" to all deleterious reproductive behavior. Although the ovaries are the major control center for the behavior associated with the estrous cycle and house follicles that produce estrogen, mares that have undergone an ovariectomy may still exhibit estrus-like behavior. This phenomenon is supported by hormone secretion from the adrenal cortex and is referred to as "paradoxical" estrous [119]. Ovariectomy is viewed as a last resort for the management of estrus behaviors due to the limitation this surgical method places on the future reproductive capacity of the mare and marginal efficacy.

Assessing a mare's behavior during winter anestrous may be the best clinical evaluation to determine if an ovariectomy can be helpful. During this time, the behavior of the mare can be evaluated while ovarian activity is minimized or absent. However, mare physiology is complex and not always consistent. During the winter months consisting of short days, some mares (10-15%) continue their estrous cycles and do not become anovulatory. In this case, it is possible the mare is exhibiting deleterious behavior

associated with a true state of estrus even during the anovulatory period of the year. If a mare's behavior is evaluated during the winter anovulatory phase and "bad" behaviors are still present, ovariectomy may consequentially make the mare's behavior worse. It is often misconstrued that exogenous progestin treatment will have the same effect as ovariectomy. This is not the case as the ovaries house the primary source of endogenous progesterone, and removing one hormone is not the same as adding a different one. A veterinarian may gain more insight by using hormonal analysis of progesterone and estrogen to determine a direct association between ovarian activity and mare behavior.

14. Pregnancy as a Method of Estrus Suppression

Pregnancy is the most common reason for prolonged CL function. To achieve luteostasis associated with pregnancy multiple events must occur. The mare must be mounted by a stallion and undergo successful copulation or be bred by artificial insemination (AI) resulting in a fertilized oocyte to undergo embryonic development. The embryo must successfully pass from the oviduct into the uterine lumen and remain mobile in the uterus, contacting the majority of the endometrial surface. The mobility of the embryo in combination with the secretion of a currently unknown substance disrupts the luteolytic pathway of the mare to prolong the function of the primary CL [87].

In the efforts of estrus suppression, it is possible to manually rupture the established conceptus by transrectal manipulation. In a study by Lefranc and Allen using 11 mares, all mares displayed CL function beyond 60 days post-ovulation when the embryo was manually reduced between 16 and 22 days post-ovulation [120]. It is also possible to abort a foal between 50 and 60 days of gestation and maintain elevated

progesterone concentrations for over 120 days post-ovulation. At this later point in gestation the mare has developed endometrial cups, which will effectively inhibit the mare from returning to estrus for approximately 60 days. Aborting a foal at a later point in gestation is much more difficult than manually reducing an early conceptus. Pregnancy as an effort to accomplish estrus suppression is not fully successful, as a small number of mares will show signs of estrous even while pregnant [109].

With the manual reduction of a conceptus and clinical abortion of a foal there are also undesirable implications. The cost of getting a mare pregnant and the ethics involved in terminating a healthy pregnancy may be objectionable to some horse owners [121].

15. Oxytocin Induced Luteal Maintenance

In the on-going effort to suppress estrus behavior in the mare, there have been many changes in the clinical "gold standard." All of the previously discussed methods have their individual benefits and deleterious consequences. Further research of estrous suppression techniques is essential to provide a reliable and applicable treatment protocol. The use of oxytocin to suppress estrus behavior in mares is gaining use among veterinarians as a clinical therapy to prolong CL function for the purposes of estrus suppression. Oxytocin is most often thought of as a catalyst of the luteolytic pathway that eliminates function of the CL, but can also be exogenously administered to disrupt the upregulation of COX-2 and prolong CL function. Spontaneously prolonged CL function has been observed in about 10% of cycling mares and has an average duration of 63 ± 15.6 (SD) days [15]. Oxytocin is relatively inexpensive and can be delivered intramuscularly in an aqueous solution. The use of oxytocin also provides a method of

estrus suppression that does not require the use of exogenous steroid hormones, which is a concern to some regarding performance horses [9].

15.1 Early Oxytocin Discoveries

In a 1987 study, Goff et al. identified the ability of exogenous oxytocin to prolong luteal function in mares. Four non-pregnant mares were administered oxytocin IV (10 IU/500 kg) daily from days 9 through 14 post-ovulation, and again on days 16, 18, and 20. Indicated by progesterone concentrations staying above 4 ng/ml for the remainder of the study, none of the treated mares returned to estrus by 22 days post-ovulation [79]. In a 1999 study, Stout et al. hypothesized that oxytocin was integral to luteolysis and determined if continuous systemic administration of oxytocin in cycling mares delayed luteolysis [31]. They approached this study by implanting subcutaneous osmotic minipumps filled with a solution of oxytocin in the neck of 10 mares. The pumps were inserted into five mares on day 8 post-ovulation and five mares on day 10 post-ovulation and delivered oxytocin at a rate of 5 μl/h. The osmotic minipumps were maintained in the mares for 12 days while mares were transrectally palpated and scanned using ultrasonography. Blood samples were collected from the mares and serum progesterone concentrations were evaluated for determination of CL function. The results indicated that four of five mares administered the minipump on day 8 post-ovulation and three of five mares administered the minipump on day 10 post-ovulation maintained elevated serum progesterone concentrations. In the group of mares administered the minipump on day 10 post-ovulation, both mares that failed to develop prolonged luteal function underwent premature luteolysis. This hastened luteolytic event is a good example of the

dual-functionality of oxytocin at the level of the endometrium. Exogenous oxytocin administration in early diestrus can work to inhibit the luteolytic process. Alternatively, exogenous administration or endogenous pulses of oxytocin in mid to late diestrus (on or after day 10) may trigger the luteolytic process. The transition from anti-luteolytic to pro-luteolytic roles of oxytocin will be discussed in more detail later in this review. In correlation to the maintenance of progesterone concentrations, the primary CL remained observable through ultrasonographic evaluation.

This research on the role of oxytocin in luteolysis helped open the door of exploration into oxytocin as a method of estrus suppression.

15.2 Twice-Daily vs Once-Daily Oxytocin Administration

To develop an oxytocin treatment protocol to prolong CL function, Vanderwall et al. treated six mares twice daily with 60 IU of oxytocin IM from days 7 through 14 [122]. Treated mares were compared to a saline treated control group to determine if luteolysis would be averted and evaluate the prolonged function of the CL. All six of the mares treated with oxytocin exhibited elevated progesterone concentrations above 1 ng/ml continuously through day 30 while none of the control mares displayed prolonged luteal function.

In a follow up study, once and twice daily IM administration of 60 IU of oxytocin were compared [10]. Eight mares were administered oxytocin once daily and seven mares were treated twice daily from day 7 through 14 post-ovulation. In addition, seven untreated control mares were used to account for spontaneous prolonged luteal function.

It was determined that there was not a significant difference in the treatment protocols with five of eight (63%) mares treated once daily and five of seven (71%) mares treated twice daily having prolonged CL function. In the control group, one of seven (14%) mares exhibited spontaneous prolonged luteal function. This study determined that once daily administration was equally efficacious to twice daily administration of oxytocin. A subsequent study showed that once daily administration becomes more efficacious with longer durations of treatment. Keith et al. treated groups of seven mares from day 8 to 10, 8 to 12, and 8 to 14, with 60 IU oxytocin IM daily and observed 43%, 57%, and 86% prolonged CL function indicating that in order to achieve an optimal effect, the oxytocin treatment must be continued until day 14 after ovulation [13].

15.3 Chronic Oxytocin Administration

Most estrus suppression oxytocin therapies require knowledge of the specific point of the estrous cycle that the mare is in. This is determined by repeated transrectal examination by a veterinarian using palpation and ultrasonography. The cost of repeated transrectal examinations can be a major drawback to mare owners. The chronic administration of oxytocin is an available solution to the owners wanting to avoid these repeated examinations. Parkinson et al. demonstrated that daily IM administration of 60 IU of oxytocin continued for 29 days can effectively prolong CL function [11]. Seven of nine mares had prolonged CL function when treatment was initiated at a random point in the estrous cycle. It was determined that three of the seven mares with prolonged CL function first underwent luteolysis before developing prolonged CL function following the subsequent ovulation. The Parkinson et al. study of chronic administration of

oxytocin clearly demonstrated the luteolytic and antiluteolytic effects of oxytocin. When treatment was initiated during late diestrus the endometrium was prepared to trigger the luteolytic process. Oxytocin at this time point interacts with the uterus by stimulating the cyclooxygenase pathway of prostaglandin synthesis. This pathway results in the production of PGF2α, which is the key hormone in luteolysis, and stops the CL from producing progesterone. Alternatively, when treatment was initiated earlier in diestrus the endometrium of the mare was unable to fulfill the luteolytic process. The inability to synthesize PGF2α was continued as the oxytocin treatment inhibited the upregulation of COX-2, an important synthetic enzyme in the cyclooxygenase pathway. The physiology of prolonging CL function using oxytocin treatment will be discussed in more detail later in this review. The anti-luteolytic and pro-luteolytic functions of oxytocin are extremely important to understand in the development of new treatment protocols and pharmacological development of oxytocin analogs. Successful chronic oxytocin administration intended to prolong luteal activity may work through one or both functions of oxytocin depending on the phase of the mare's estrous cycle.

15.4 Proprietary Slow-Release Oxytocin

Although the above-mentioned oxytocin protocols are effective, the requirement for daily administration is a limitation to their clinical application. With a half-life of 6.5 minutes, the pharmacological characteristics of oxytocin also pose a challenge in the development of a therapeutic regimen that does not require daily administration. Proprietary slow-release oxytocin (SR-OT) formulations have been explored in pilot studies and will be expounded upon in the research associated with this review. In a 2018

preliminary study, three different SR-OT formulations from SR Veterinary Technologies (Windsor, CO) were compared. Two of the slow-release formulations were administered only once on day 7 and the third formulation was administered once on day 7 and again on day 10. The third formulation, with administration on day 7 and 10 yielded the greatest efficacy with five of six mares treated having prolonged CL function [12]. All pilot study preparations contained 2,400 IU/ mL and were administered in one mL doses. It was determined that more research must be conducted to validate the efficacy of this proprietary formulation and protocol in comparison to a control group which is discussed in the research associated with this review.

15.5 Oxytocin Administration Indicated by Behavior

The limitations of repeated examinations to determine estrous cycle status pose a financial and time-consuming barrier in front of most oxytocin therapies. To avoid transrectal examination, a mare can be evaluated for estrus and diestrus behaviors to time the initiation of treatment. In a study by Manning et al. 54.5% of mares had prolonged CL function when 60 IU of oxytocin was administered IM on day 8 through 17 after the first day the mare showed behavioral estrus when teased to a stallion [123]. Although this study indicated efficacy at a lower rate compared to protocols using transrectal ultrasound and palpation (70-75%), it shows that it is possible to avoid the costs associated with veterinary examinations.

15.6 Oxytocin Analogs

Carbetocin, an oxytocin analog, was hypothesized to have a similar effect to

oxytocin in attempts to prolong CL function. Carbetocin has a half-life almost triple that of aqueous oxytocin (17.2 minutes in comparison to 6.8 minutes) [124,125]. The efficacy of carbetocin (1.19 mg) was compared to oxytocin (60 IU) in a 2013 study by Bare et al. Both treatments were administered IM from day 7 to 14 post-ovulation, but the carbetocin treatment significantly abbreviated the IOI rather than lengthening it [126]. An interesting note to add from the study by Bare et al. is that neither treatment (carbetocin or oxytocin) was reported to inhibit estrous behavior when mares were exposed to a mature stallion.

16. Physiology of Prolonging CL Function

Understanding the physiology of prolonging CL function has been a puzzling research task. There are many pieces to the process of luteolysis which are all necessary for successful termination of luteal funtion. The methods described above which prolong luteal activity all disrupt luteolysis at one point or another. It has been hypothesized that the downregulation of oxytocin receptors, the inability for the endometrium to synthesize prostaglandin, or the inability for the endometrium to secrete prostaglandins. Recent studies have shown that oxytocin therapies as well as IUD therapies inhibit the upregulation of COX-2 in order to disrupt the luteolytic pathway [13, 77].

COX-2 is a catalytic enzyme which influences the synthesis of PGH2 from AA, after which PGH2 is subsequently converted into multiple prostaglandins including PGF2α. In cattle, bovine interferon-tau associated with pregnancy also causes the down-regulation of COX-2 and prostaglandin F synthase expression [92]. Pregnancy in mares also inhibits COX-2 expression [77]. It is known that pregnancy delays the upregulation

of oxytocin receptor expression and that the conceptus plays a role in reducing the number oxytocin receptors expressed [76,82]. The use of oxytocin to prolong CL function has been hypothesized to inhibit oxytocin receptor expression as well. This hypothesis was disproved by Vanderwall et al. as oxytocin-binding capacity on day 15 post-ovulation was not significantly different in normal cycling mares in comparison to mares treated with 60 IU oxytocin IM twice daily from day 7 to 14 post-ovulation [10]. The specific mechanism by which oxytocin or IUD therapies disrupt COX-2 has not been determined. To further understand the mechanism by which these techniques inhibit luteolysis it may be helpful to look at the steps previous to COX-2 in the luteolytic pathway. Rivera et al. hypothesized that PLA2, which is required for releasing AA from membrane cells, could initially be obstructed by an IUD [77].

Reports have indicated that IUD techniques can be administered at any point in the estrous cycle and exhibit successful disruption of luteolysis [116]. The same cannot be said for oxytocin therapies in which only the chronic administration of aqueous oxytocin has shown to be efficacious when it was initiated at any time point of the estrous cycle [11]. In order to properly time other oxytocin protocols, it is important to understand that oxytocin plays a role in luteolysis during late diestrus [50]. To avoid the luteolytic effect of oxytocin, treatment must be administered before day 10 post-ovulation. As mentioned previously, treatment must not only be initiated before day 10, but must be continued until day 14 to increase efficacy and inhibit COX-2 [13]. In a 2013 study, Keith et al. researched many aspects of oxytocin treatment to prolong luteal function [13]. Their first experiment demonstrated the necessity of oxytocin treatment

beyond day 10 post-ovulation to successfully prolong CL function. Three of seven, four of seven, and six of seven mares exhibited prolonged luteal activity after daily intramuscular oxytocin administration of 60 IU on days 8 to 10, 8 to 12, and 8 to 14 post-ovulation, respectively. In a second experiment from the same publication, day 14 uterine biopsies from mares treated with oxytocin from days 8 to 14 were compared to day 14 uterine biopsies from saline-treated control mares. The uterine biopsies were evaluated for prostaglandin synthetic enzyme expression, as well as oxytocin and estrogen receptor expression. Keith et al. found that mares treated with oxytocin expressed a significantly lower amount ($p < .01$) of COX-2 than the control mares with a 3.7-fold decrease in oxytocin-treated mares. It was also determined that there was no significant difference in PGFS, estrogen receptor, or oxytocin receptor expression between the oxytocin-treated mares and control mares. This study by Keith et al. provided information vital to the understanding of oxytocin techniques to prolong luteal activity and was influential on the research associated with this review.

The similarities between pregnancy and therapeutically prolonged CL function provoke interest into their comparative research. Of course, one of the greatest puzzles of all animal science is the complexity of maternal recognition of pregnancy in the mare. The physiological understanding of estrus suppression therapies may give clues towards the recognition of new factors pertaining to maternal recognition of pregnancy.

CHAPTER III

EVALUATION OF A PROPRIETARY SLOW-RELEASE OXYTOCIN FORMULATION ON CORPUS LUTEUM FUNCTION IN MARES[1]

ABSTRACT

Prolonging function of the corpus luteum (CL) is a method of suppressing estrus that relies on continued secretion of endogenous progesterone to keep mares out of heat naturally. The use of oxytocin treatment to prolong CL function is gaining increasing use, and the most common treatment protocol involves administration of 60 units of oxytocin intramuscularly (IM) once daily on days 7 to 14 after ovulation (8 daily treatments). Although that protocol induces prolonged CL function in ≥70% of treated mares, the need for daily administration is a drawback to its use. Therefore, the objective of this study was to evaluate the efficacy of a proprietary slow-release oxytocin formulation (SR-OT) for prolonging CL function that requires only two treatments. Mares were examined via transrectal palpation and ultrasonography to determine the day of ovulation (day 0) and then randomly assigned to a non-treated control group and an SR-OT treatment group (n = 8 mares/group). Mares in the treated group received 1.0 mL of SR-OT containing 2,400 IU oxytocin IM once on Day 7 and again on Day 10 after ovulation. Jugular blood samples were collected on day 0 and then every M, W and F for 50 days for determination of the serum progesterone concentration. Mares were classified as having prolonged CL function if their progesterone concentration remained

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>1.0 ng/mL continuously for at least 30 days. Corpus luteum function was prolonged in 0/8 (0%) control mares and 6/8 (75%) of the SR-OT-treated mares ($p < 0.01$). The demonstrated efficacy of this two-injection, SR-OT protocol represents a 75% reduction in the number of oxytocin treatments compared to daily administration of oxytocin from Day 7 to 14, making it a more practical treatment protocol.

1. Introduction

Suppression of estrous behavior is a common management strategy implemented in mares. Various methods to suppress estrous behavior are used to mitigate real and/or perceived negative effects of the behavior on performance activities such as showing and racing [1]. Although daily administration of the FDA-approved, orally-active, synthetic progestin altrenogest is efficacious and has been widely used for suppressing estrous behavior in mares, the expense, need for long-term daily administration, and safety risks for personnel during handling have collectively led to the increased use of alternative methods of suppressing estrus [5]. For the past 15 years, placement of an intrauterine glass ball has been the most common method of prolonging corpus luteum (CL) function [8]. However, the use of intrauterine glass balls has been called into question due to numerous reports of severe complications associated with their use [7]. Prolonging the functional lifespan of the CL using exogenous oxytocin therapy is a simple and cost-effective method of suppressing estrus that has gained increased use in clinical practice. This method capitalizes on continued natural secretion of progesterone from the CL to eliminate estrous behavior [121]. Sustaining the CL using oxytocin to inhibit luteolysis has been proven effective by two different methods [9]. If the day of ovulation is known,

60 IU of oxytocin can be administered intramuscularly (IM) on days 7 through 14 after ovulation. If the day of ovulation is unknown, 60 IU of oxytocin can be administered IM for 29 consecutive days. Although both protocols provide equivalent efficacy, with an induced rate of prolonged CL function of approximately 70%, the need for daily administration is a limitation to this method of estrus suppression.

In an effort to eliminate the need for daily administration of oxytocin, the use of proprietary slow-release oxytocin (SR-OT) formulations have been investigated for prolonging CL function. Based upon the results of a previous study [12], it was hypothesized that a two-injection SR-OT protocol would deliver an appropriate amount of oxytocin for a sufficient duration of time to inhibit luteolysis. The previous study compared the efficacy of three proprietary SR-OT formulations for prolonging CL function [12]. Of the three formulations tested, one appeared to be suitably efficacious, since it prolonged CL function in 83% of the treated mares. This particular formulation involved administration of 2,400 IU oxytocin IM once on day 7 and again on day 10 after ovulation. However, that formulation was not directly compared to a control group of mares. Therefore, the objective of this study was to determine if IM administration of 2,400 IU of SR-OT once on days 7 and 10 after ovulation would prolong CL function in treated mares compared to a non-treated control group.

2. Materials and Methods

2.1. Animal Care and Use

All animal procedures were approved and conducted following the guidelines of the Utah State University Institutional Animal Care and Use Committee (Protocol

#2853). This study used 16 American Quarter Horse-type mares ranging from 3 to 18 years of age with known reproductive and health histories. Each mare used in this study was evaluated and determined to have a normal estrous cycle prior to inclusion in the study.

2.2. Experimental Protocol

Between May and July in the Northern Hemisphere (41.7° N) cycling mares were examined using transrectal palpation and ultrasonography. Mares with an ovarian follicle ≥ 30 mm in diameter and prominent endometrial edema were examined daily until ovulation (day 0) was detected. Ovulation was determined by the disappearance of a follicle > 35 mm in diameter with the resulting CL being observed by transrectal ultrasonography. Following ovulation, mares were randomly assigned to a non-treated control group (n = 8) and an SR-OT treatment group (n = 8). SR-OT mares received 1.0 mL (2,400 IU) of a proprietary SR-OT formulation (SR Veterinary Technologies, Windsor, Colorado 80550) IM once on day 7 and once on day 10 post-ovulation.

2.3. Blood Collection

Using red-top blood collection tubes with no anti-coagulant, blood samples from the jugular vein were collected on day 0 and then every Monday, Wednesday, and Friday through and including day 50. Blood samples were allowed to clot at room temperature before being centrifuged at 125 × g for 20 minutes at 21° C. Serum was recovered from each individual sample and stored at -20° C until it was analyzed for progesterone concentration.

2.4. Progesterone Assay

Serum samples were processed and evaluated for progesterone concentration by a commercially available kit (Immulite Progesterone, Siemens, Malvern, PA, USA) according to the manufacturer's instructions. The sensitivity of this assay was 0.2 ng/mL. Intra- and inter-assay coefficients of variation were 21.2% and 19.8%, respectively. Mares with serum progesterone levels that remained > 1.0 ng/mL for at least 30 days were considered to have prolonged CL function.

2.5. Statistical Analysis

The proportion of mares with prolonged CL function in each group was compared using Fisher's Exact Test (GraphPad Software, Inc., La Jolla, CA, 92037). A value of $p < 0.05$ was considered significant.

3. Results

Corpus luteum function was prolonged in 0/8 (0%) non-treated control mares and 6/8 (75%) of SR-OT treated mares (Fig. 6; $p < 0.01$).

4. Discussion

Oxytocin has opposing effects at different stages of the estrous cycle. In late diestrus (after day 10), oxytocin (endogenous or exogenous) has a "pro-luteolytic" effect that stimulates prostaglandin-F2α (PGF2α) secretion from the endometrium. Luteolysis occurs during late diestrus as a result of endogenous pulsatile oxytocin secretion from the posterior pituitary gland that is associated with pulsatile secretion of uterine PGF2α [68, 127]. The luteolytic mechanism may be initiated by uterine derived oxytocin and

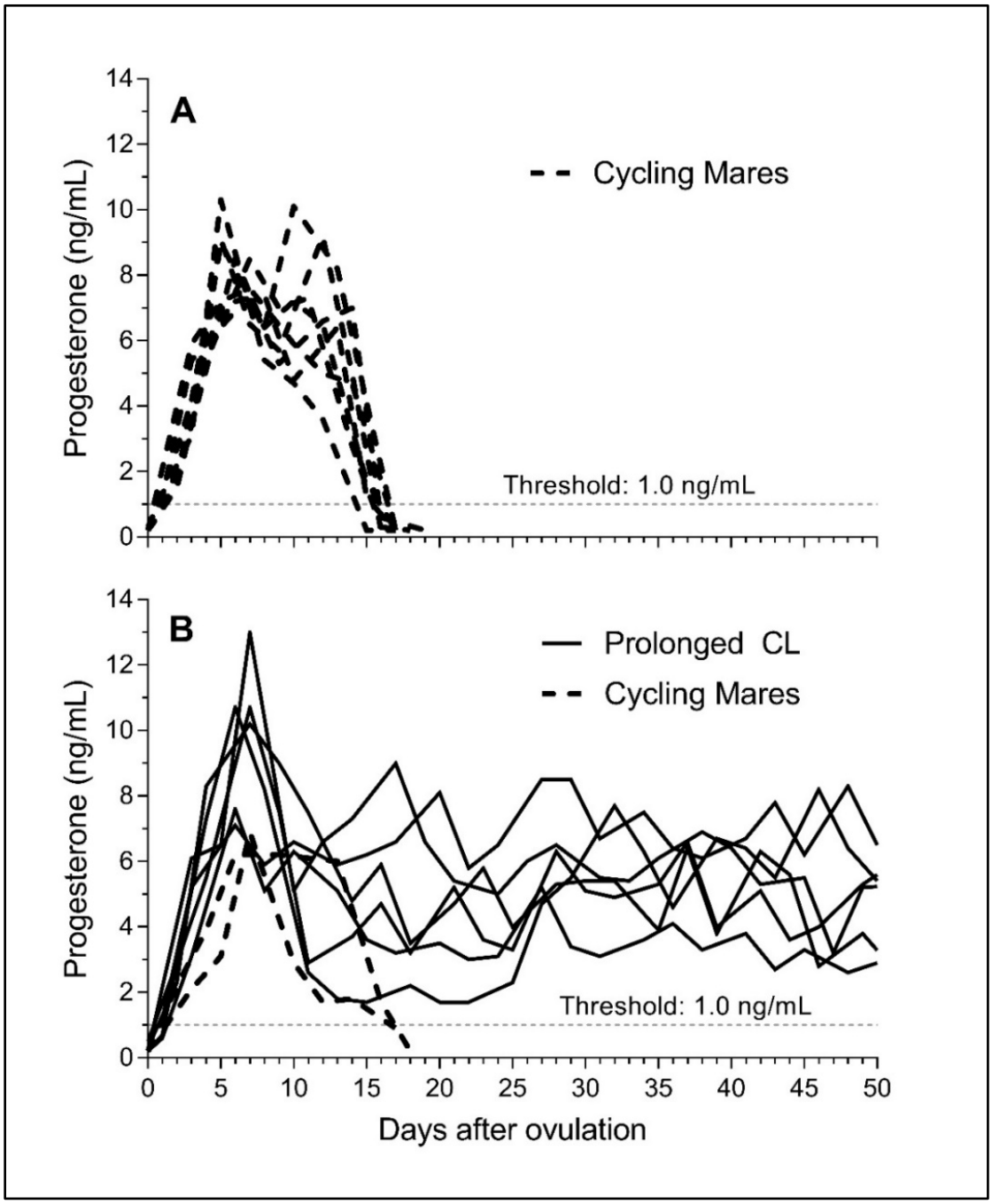

Fig. 6. Progesterone concentration profiles of non-treated control mares and mares treated with a proprietary slow-release oxytocin (SR-OT) formulation. (A) Serum progesterone concentrations in 8 non-treated control mares. (B) Serum progesterone concentrations in 8 mares treated intramuscularly with 1.0 mL of a proprietary SR-OT formulation containing 2,400 IU on days 7 and 10 after ovulation. For clarity, lines are truncated after luteolysis in mares that did not have prolonged CL function. All mares that underwent luteolysis did so before 18 days post-ovulation. A threshold for progesterone concentration, indicating continued CL function, is marked at 1.0 ng/mL.

continues through positive feedback mechanisms involving PGF2α to stimulate posterior pituitary release of oxytocin [128]. Prior to day 10 after ovulation, therapeutic treatment with oxytocin has an "anti-luteolytic" effect as it inhibits the upregulation of the key synthetic enzyme necessary for PGF2α production, cyclooxygenase-2 (COX-2) [13,129]. Whether the instigating factor is therapeutic or idiopathic, the functional life span of the CL in the absence of a luteolytic event is approximately 2 to 3 months [15]. Although spontaneously prolonged luteal function did not occur in any of the control mares of this study, this event can occur in up to 10% of mares during the breeding season and 25% of mares in the fall transition [33,36]. This idiopathic phenomenon of spontaneously prolonged luteal function highlights the need for an appropriate group of control mares when evaluating the effect of any treatment on luteal function.

Therapeutically prolonging the functional lifespan of the CL in mares using oxytocin to inhibit luteolysis has proven to be an effective method of estrus suppression that provides an alternative to daily administration of oral progestins (e.g., altrenogest) [9, 121]. It is assumed that SR-OT has a short-term continuous effect similar in function to the previously mentioned 8-day protocol (days 7 through 14) in the manner that it occupies oxytocin receptors at the endometrial epithelium and inhibits upregulation of COX-2. In a study by Keith et. al. comparing daily administration of 60 IU of aqueous oxytocin IM on days 8 to 10, 8 to 12, and 8 to 14, the proportion of mares with prolonged luteal function increased significantly as the duration of treatment increased [13]. Therefore, continuation of oxytocin treatment beyond day 12 after ovulation is required to attain maximum efficacy in the inhibition of upregulation of COX-2 [13]. The efficacy

of SR-OT administered once on day 7 and again on day 10 demonstrates that there was a sufficient amount of oxytocin present to inhibit the upregulation of COX-2. Therefore, delivery of oxytocin via administration of SR-OT on days 7 and 10 had an effect equivalent to the daily administration of aqueous oxytocin on days 7 to 14.

The present results imply that SR-OT treatment delivers oxytocin at an appropriate therapeutic level from day 7 through 10, and again from day 10 through 13, resulting in an optimal anti-luteolytic effect without having pro-luteolytic consequences. The same is not true for the long-acting oxytocin analogue carbetocin. Carbetocin has a half-life of 17.2 minutes, in comparison to the 6.8-minute half-life of aqueous oxytocin [124,125]. In a study comparing the efficacy of carbetocin (1.19 mg) compared to aqueous oxytocin (60 IU) both administered IM daily from days 7 to 14, carbetocin significantly shortened the inter-ovulatory interval [126]. The lack of carbetocin efficacy contrasted by the successful inhibition of luteolysis with SR-OT in the present study implies that SR-OT delivered enough oxytocin to inhibit luteolysis, without triggering pro-luteolytic effects.

In conclusion, IM administration of SR-OT containing 2,400 IU oxytocin on days 7 and 10 after ovulation was reliably efficacious for prolonging the functional lifespan of the CL in mares. Therefore, this new treatment protocol is equally effective when compared to the previously established eight-injection protocol using daily oxytocin treatments on days 7 to 14 after ovulation [10]. Importantly, this two-injection oxytocin protocol reduces the number of required treatments by 75% making this method more clinically practical.

CHAPTER IV

OXYTOCIN INDUCED SECRETION OF 13,14-DIHYDRO-15-KETO PROSTRAGLANDIN F2α (PGFM) IN MARES WITH PROLONGED CORPUS LUTEUM (CL) FUNCTION[2]

ABSTRACT

Therapies using hormonal or physical stimuli to prolong CL function have gained popularity as tools for estrus suppression in performance mares. Previous studies have shown that the endometrium of mares having been treated with either oxytocin or an intrauterine device (IUD) as methods to prolong CL function disrupt the synthesis and secretion of prostaglandin F2α (PGF2α). In these therapies, upregulation of cyclooxygenase-2 is inhibited and impairs PGF2α production. This study evaluated the ability of the endometrium to synthesize and secrete PGF2α in mares with therapeutically-induced prolonged CL function. After ovulation (day 0), CL function was evaluated using progesterone concentrations in venous blood samples. Prolonged CL function was induced using oxytocin treatment, after which mares were assigned to groups 50-59 days, 60-69 days, or 70-79 days (50s, 60s, or 70s) post-ovulation. For comparison, a 14-day post-ovulation control group was used. The ability of the endometrium to synthesize PGF2α was evaluated by measurement of 13,14-dihydro-15-keto PGF2α (PGFM) in response to a single 10 IU intravenous oxytocin bolus (time 0). Blood samples were collected at -30, -15, 0, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, and

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120 minutes. The area under the curve was significantly higher in the 70s group compared to the 50s and 60s groups ($p< 0.001$; $p< 0.02$, respectively). There was no significant difference between the 70s group and the control group ($P>0.36$). The progesterone concentration curves are consistent with these findings as CL function was maintained after the oxytocin bolus in 4 of 4 mares in the 50s, 3 of 4 mares in the 60s, and 0 of 3 mares in the 70s group. These results revealed a clear shift in the endometrial environment of the mare between 50 and 70 days after ovulation concerning the ability to synthesize PGF2α during the period of prolonged CL function.

1. Introduction

Endogenous oxytocin in the mare is a primary stimulus that provokes the synthesis and secretion of the luteolytic hormone prostaglandin F2α (PGF2α) from the endometrium. Exogenous oxytocin, alternatively, can be therapeutically administered for both luteolytic and anti-luteolytic functions depending on the phase and timing of the mare estrous cycle when it is administered [79,121]. At the writing of this article, it has been known for over 20 years that oxytocin can therapeutically prolong corpus luteum (CL) function in cycling mares [31]. The clinical use of oxytocin to inhibit luteolysis has gained popularity as an alternative estrus suppression therapy because of the simple and cost-effective treatment protocols that are more efficacious and safer than previous methods to prolong CL function (i.e., intrauterine glass balls) [7].

Luteolysis is a critical point in the mare estrous cycle at which progesterone concentrations corresponding to the function of the CL decrease to less than 1.0 ng/mL [15]. Complete luteolysis in the mare involves many pulses of endometrial derived

PGF2α preceded by pulses of oxytocin originating from the posterior pituitary gland over a 23-hour period [53]. Oxytocin interacts with its receptors at the endometrium to stimulate luteolytic surges of PGF2α targeting the CL through systemic circulation [130]. The state of prolonged CL function in the mare is described as progesterone concentrations greater than 1.0 ng/mL for more than 30 days post-ovulation. In a state of prolonged luteal activity, the average duration of CL function is 63 days (SD $\pm$ 15.6 days) [15]. Two protocols using exogenous oxytocin have been described for prolonging CL function. One protocol involves administering 60 IU of oxytocin intramuscularly (IM) once daily on days 7 through 14 after ovulation [10]. The second protocol can be initiated at any point in the estrous cycle and involves daily administration of 60 IU of oxytocin IM for 29 consecutive days [11]. Both of these protocols are equally effective and prolong CL function in about 70% of treated mares.

In an effort to extend the average length of prolonged CL function using oxytocin treatment, the addition of human chorionic gonadotropin (hCG) to oxytocin treatment has been studied. In an experiment by Vanderwall et al., mares were first treated with 60 IU of oxytocin IM to prolong CL function on days 7 through 14 after ovulation [14]. It was hypothesized that hCG, administered on days 30, 45, 60, 75, and 90 would have a luteotrophic effect, or induce the ovulation of a diestrus follicle. It was thought that a diestrus ovulation would reset the length of prolonged CL function and the new CL formed from the ovulatory transition of the diestrus follicle would persist for an additional 65 days. The results indicated that there was no significant difference in the length of luteal function between mares that were treated with oxytocin to prolong luteal

function and mares that received hCG in addition to oxytocin treatment. Upon further evaluation of the records, it was apparent that even when a diestrus ovulation resulted in a functional CL, the duration of elevated progesterone concentrations was not extended. This event demonstrated the apparent return of the luteolytic mechanism in mares around the described length of average prolonged luteal function.

This study evaluated the ability of the endometrium in mares in a state of prolonged luteal activity to synthesize and secrete PGF2α. The main metabolite of PGF2α is 13,14-dihydro-15-keto-prostaglandin F2α (PGFM), which has a longer half-life and is representative of circulating concentrations of PGF2α [131]. In the development and refinement of estrus suppression therapies, it is important to understand the dynamic physiology of the mare endometrium. By analyzing the concentrations of PGFM in response to an oxytocin bolus in mares at different time-points of prolonged luteal activity, the hypothesized return of the luteolytic pathway can be assessed.

2. Materials and Methods

2.1 Animal Care and Use

All animal procedures were approved and conducted following the guidelines of the Utah State University Institutional Animal Care and Use Committee (Protocol #10079). This study used 14 American Quarter Horse-type mares ranging from 3 to 18 years of age with known reproductive and health histories. Before inclusion in this study each mare was evaluated by transrectal palpation and ultrasonography and determined to have a physiologic estrous cycle.

2.2 Experimental Protocol

Between May and September in the Northern Hemisphere (41.7° N), cycling mares were examined using transrectal palpation and ultrasonography. Mares with an ovarian follicle ≥ 30 mm in diameter and prominent endometrial edema were examined daily until ovulation (day 0) was detected. Ovulation was defined as the disappearance of a follicle > 35 mm in diameter with the resulting CL being observed by transrectal ultrasonography. Following ovulation, mares were treated with one of two oxytocin-based methods of prolonging luteal function. Three mares were treated with 2400 IU of a proprietary slow-release oxytocin administered IM on days 7 and 10 after ovulation as described by a previous publication [132]. Eight mares were treated from day 7 through 14 with a single daily injection of 60 IU of aqueous oxytocin IM (Oxytocin, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) following a protocol previously described by Vanderwall et al. [10]. Mare serum was evaluated for progesterone concentrations to determine prolonged luteal function. After mares were determined to have luteal activity for longer than 30 days, they were assigned to groups at 50-59 (50s: n = 4), 60-69 (60s: n = 4), and 70-79 (70s: n = 3) days post-ovulation to undergo an exogenous "oxytocin challenge." A control group (n = 3) was untreated and underwent the oxytocin challenge at 14 days post-ovulation to replicate an expression of PGFM at a physiologically normal time point.

2.3 Blood Collection

Using red-top blood collection tubes with no anticoagulant, blood samples from the jugular vein were collected on day 0 and every Monday, Wednesday, and Friday until

one week following the oxytocin challenge date. Red-top blood collection tubes were allowed to clot at room temperature before being centrifuged at 125 × g for 20 minutes at 21° C, after which serum was recovered from each sample and stored at -20° C until it was analyzed for progesterone concentration. Jugular blood samples during oxytocin challenges were collected in green-top tubes with sodium heparin. Green-top samples were immediately placed on ice until centrifuged at 1000 × g for 10 minutes at 5° C. Plasma was then recovered from the green-top samples and stored at -20° C until it was analyzed for PGFM concentration.

2.4 Oxytocin Challenge

A single bolus of 10 IU of aqueous oxytocin was given IV at time 0. Blood samples were collected 30 and 15 minutes prior to the oxytocin bolus, time 0 was collected immediately before oxytocin administration and subsequent sampling at 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, and 120 minutes after oxytocin administration. All mares were evaluated using transrectal ultrasonography immediately before the oxytocin challenge began to visually assess CL presence and endometrial edema.

2.5 Hormone Assays

2.5.1 Progesterone Assay

Serum samples were processed and evaluated for progesterone concentration using a commercially available kit (Immulite Progesterone, Siemens, Malvern, PA, USA) according to the manufacturer's instructions. The sensitivity of this assay was 0.2 ng/mL. Intra-assay and inter-assay coefficients of variation were 11.6% and 14.6%, respectively.

Mares were considered candidates for the oxytocin challenge only if levels of progesterone concentration remained > 1.0 ng/mL until the date of the oxytocin challenge.

2.5.2 PGFM Assay

Plasma samples were evaluated for 13,14-dihydro-15-keto-prostaglandin F2α using a commercially available kit (13,14-dihydro-15-keto-prostaglandin F2α Elisa Kit, #516671, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. This assay was previously validated by Keith, et. al. [13]. The sensitivity of this assay was 16.3 pg/mL. Intra-assay and inter-assay coefficients of variation were 14.1% and 17.7%, respectively.

2.6 Statistical Analysis

PGFM concentrations were compared by evaluating the interaction of the area under the curve and the treatment groups using a one-way Analysis of Variance (ANOVA) and the Tukey-Kramer method of multiple comparisons. Values of area under the curve (AUC) were normalized before evaluation by ANOVA by a Log10 transformation. The maintenance of luteal function was compared to the PGFM area under the curve using an unpaired t-test to provide supporting statistics. All statistical analyses were performed using GraphPad Prism (GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla, California, USA). A P value of $P \leq 0.05$ was considered significant.

3. Results

The concentration of PGFM was higher in the 70s group than in the 50s group ($p < 0.001$) and 60s group ($p = 0.01$) in response to an oxytocin bolus of 10 IU (Fig. 7 and Fig. 8). The concentration of PGFM following oxytocin was not significantly different comparing the control group to the 70s group ($p = 0.36$), or between the 50s group to the 60s group ($p = 0.23$) (Fig. 8). The concentrations of PGFM among all groups compared using an ANOVA model were significantly different ($p > 0.006$). The maintenance or loss of luteal function following the oxytocin challenge was dependent on the endometrial response of concentration of PGFM during the oxytocin challenge ($p < 0.001$; see Fig. 9 and Fig. 10). Progesterone concentration was maintained above 1 ng/mL in 4/4 (100%) of mares in the 50s group, 3/4 (75%) of mares in the 60s group, and 0/3 (0%) of mares in the 70s group following the oxytocin challenge (Fig. 10). In a comparison of the proportion of treated mares with prolonged CL function and mares who underwent luteolysis, the abundance of PGFM (AUC) during the oxytocin challenge significantly influenced the occurrence of luteolysis ($P = 0.023$). The average peak concentration of PGFM for the control, 50s, 60s, and 70s groups during the oxytocin challenge was 1221 pg/mL, 283 pg/mL, 567 pg/mL, and 1769 pg/mL, respectively.

4. Discussion

Extending the functional span of the CL has gained increased use as a method of estrus suppression in mares. There are many techniques that can be therapeutically implemented to achieve this goal including the use of IUDs and oxytocin therapies [1]. These clinical tools have an influential effect on the synthetic enzymes for PGF2α,

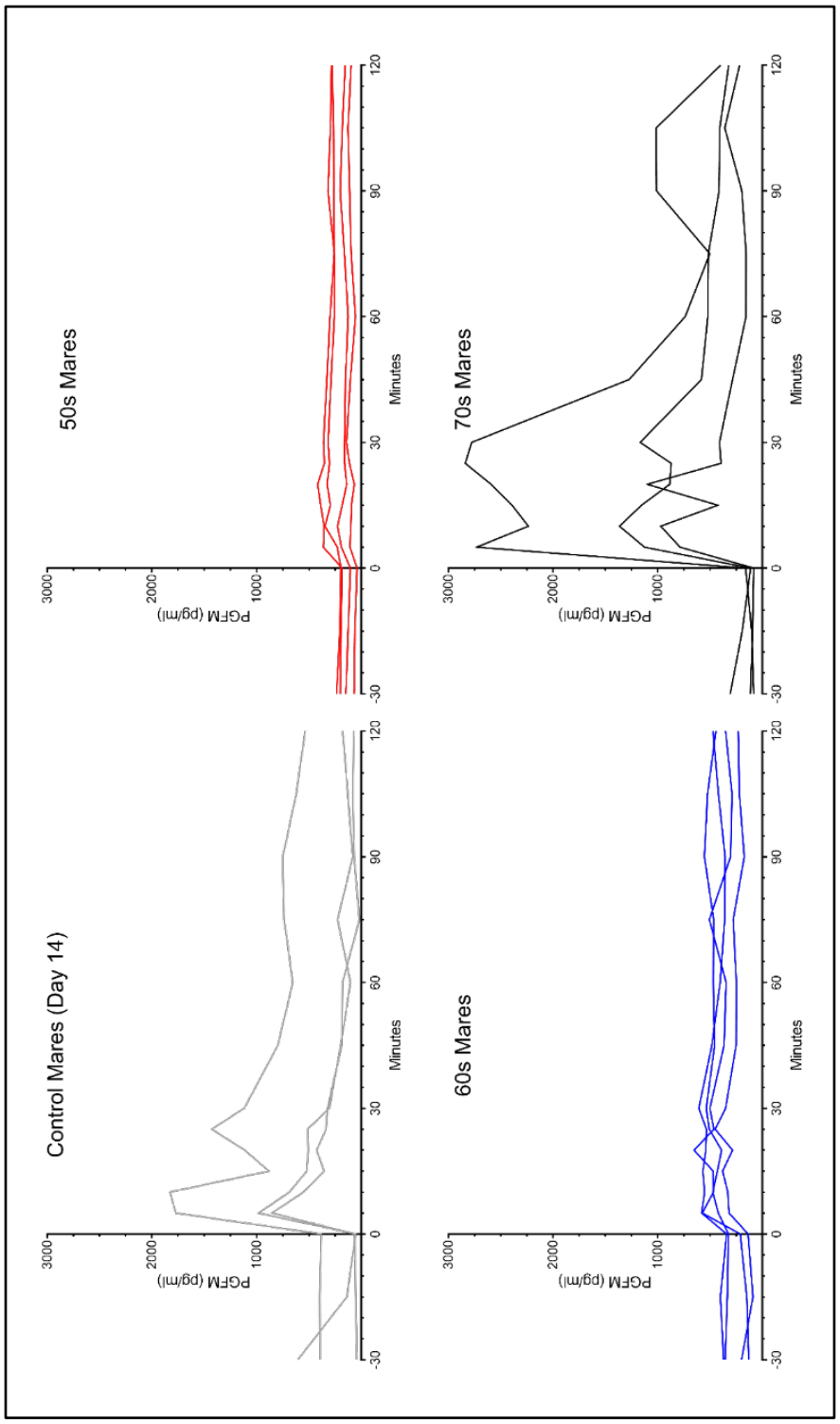

Fig. 7. Plasma concentrations of of 13,14-dihydro-15-keto prostaglandin F2α in mares treated at 14 (control), 50-59, 60-69, and 70-79 days post ovulation, with an intravenous dose of 10 IU oxytocin.

Tukey Kramer Multiple Comparisons Test*	
Control vs. 50s	P= 0.01
Control vs. 60s	0.22
Control vs. 70s	0.36
50s vs. 60s	0.23
50s vs. 70s	< 0.001
60s vs. 70s	0.01

*Values of area under the curve were normalized by logarithmic transformation from the time of oxytocin injection (0) until 60 minutes.

Fig. 8. Chart of multiple comparisons of PGFM, made by statistical analysis using Tukey Kramer test for multiple comparisons from an analysis of variance model.

creating a gap in the pathway to trigger luteolysis. The inhibition of COX-2 by using oxytocin or certain IUDs has been shown to be an effective method to maintain CL function [13,77]. Continued production of progesterone from prolonged luteal activity remains above 1 ng/mL until an average of 65 days post-ovulation [15]. The results of this study reveal that there is a return of the luteolytic mechanism coinciding with the average length of prolonged luteal function.

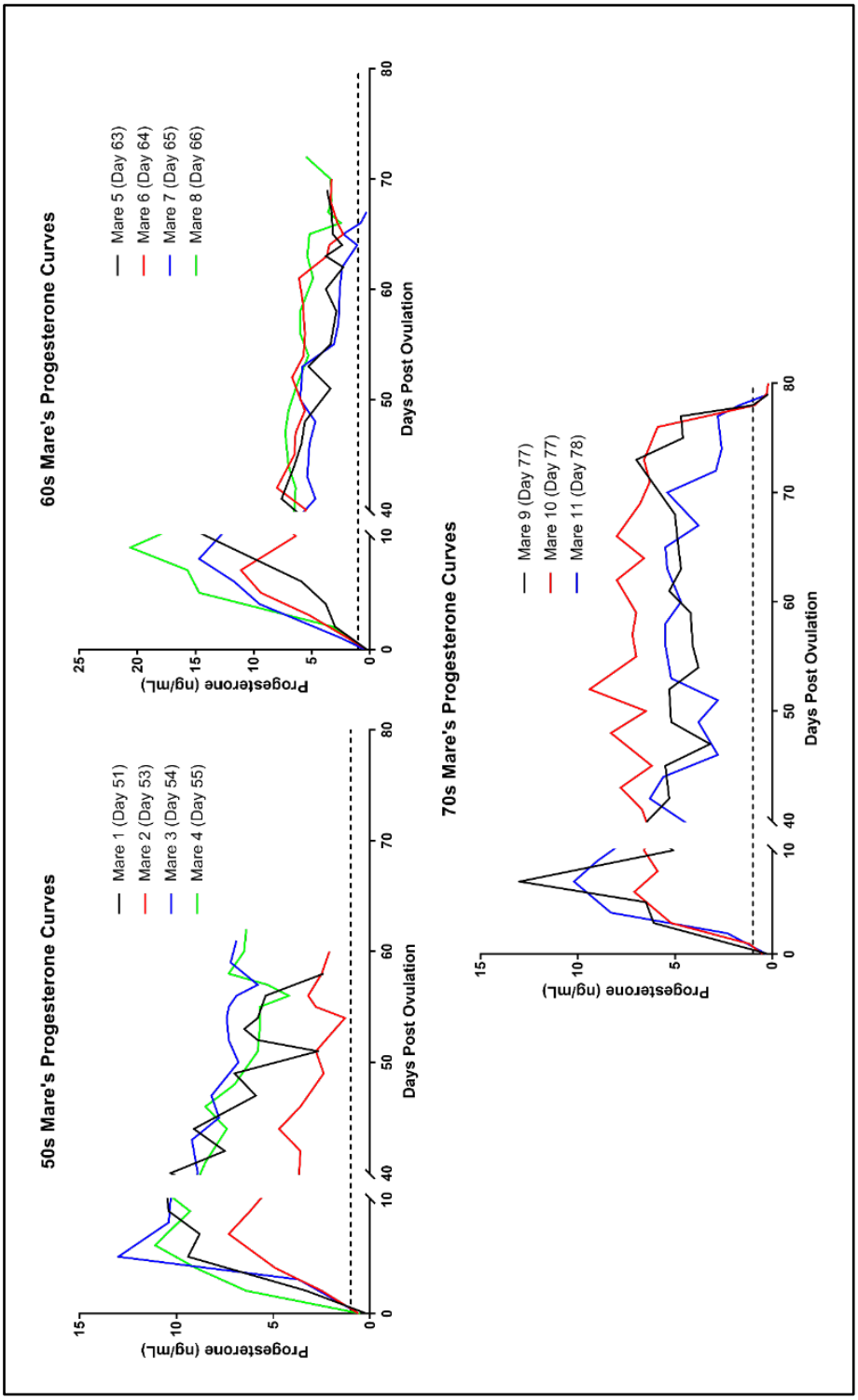

Fig. 9. Serum progesterone concentrations in mares treated with oxytocin therapies to prolong CL function. For clarity, lines are truncated after physiological determined luteolysis. A threshold for progesterone concentration, indicating continued CL function, is marked at 1.0 ng/mL.

Mares with Progesterone Concentrations Decreasing <1 ng/mL*	
50s	0/4
60s	1/4
70s	3/3

*All mares who underwent luteolysis did so within 48 hours of oxytocin administration.

Fig. 10. Proportions of mares treated with oxytocin therapies to prolong CL function who underwent luteolysis after receiving a bolus of 10 IU of oxytocin administered intravenously.

A robust increase in PGFM concentrations in response to an oxytocin bolus suggests that; (1) the oxytocin receptor is present and expressed on the endometrium, (2) secondary synthetic enzymes (COX-2) are present to produce PGF2α, and (3) PGF2α is produced and systemically circulated in great enough quantities to achieve luteolysis. Six of seven mares 50-69 days post-ovulation did not show a robust response of PGFM concentrations after a bolus of 10 IU of oxytocin. The lack of a PGFM response indicates that one or more of the components of the luteolytic process outlined above are not present and/or functional at this time. This lack of PGF2α secretion corresponds with post-oxytocin progesterone concentrations as all mares 50-59 days, and 3 of 4 mares 60-69 days post-ovulation maintained progesterone concentrations > 1 ng/mL for one week after oxytocin administration. Alternatively, all mares 70-79 days post ovulation showed a robust spike in PGFM concentrations in response to a 10 IU oxytocin bolus and failed to maintain progesterone concentrations soon after the oxytocin challenge. All mares in

this study that underwent luteolysis, following the oxytocin bolus, did so within 48 hours. The profile of diminishing progesterone concentrations after the oxytocin challenge is similar to other studies regarding luteolysis [53].

For comparison, the primary CL in pregnant mares stands alone as the main ovarian producer of progesterone for about 40 days. At this time, equine chorionic gonadotropin (eCG), synthesized by endometrial cups of the placenta, has a luteotrophic effect on the ovaries and influences the ovulation and development of secondary and accessory corpora lutea [23]. In the non-pregnant mare with prolonged luteal function, this study has shown that there is a return of the luteolytic mechanism between 50 and 70 days after ovulation. Oxytocin therapies to prolong luteal function in non-pregnant mares and the establishment of pregnancy share some similarities. In both events, there is an inhibition of COX-2 [13,76]. More research is needed to understand how either event disrupts the expression of COX-2. In pregnancy, there is continual stimulus which may play a role on the later expression of luteolytic pathway components. The addition of supplemental corpora lutea and the feto-placental unit as a source of progestins allows for a continual source of progesterone secretion from the time of initial luteinization of the primary CL to full term [23]. It has been shown in pregnant mares that there is a PGFM response to oxytocin beginning at around day 18 after ovulation [82]. In the normal-cycling non-pregnant mare, it has been shown that PGFM response induced by oxytocin peaks earlier, during the luteolytic time frame [79]. The present study indicates that the PGFM response to oxytocin in mares therapeutically treated with described oxytocin estrus suppression protocols is delayed until beyond 60 days after ovulation. The first key

step to oxytocin stimulated prostaglandin release is the ability of oxytocin to bind to its receptor at the endometrium. In mares treated with oxytocin therapies for estrus suppression, there is no significant difference in oxytocin binding capacity to its receptor and one study has shown that there is an elevated level of oxytocin receptor expression in comparison to a control group [10,129]. In pregnant mares, oxytocin receptor expression is similar to non-pregnant mares on day 12, but is significantly lower in comparison on day 14 post-ovulation [91].

There are many different techniques exploited for estrus suppression in mares. The extension of CL function relies on the interruption of the luteolytic mechanism. In efforts to achieve a more clinically applicable method of extending CL function, understanding the return of the luteolytic mechanism in a state of prolonged luteal activity is very important. Blocking the return of the luteolytic mechanism could allow for continued secretion of progesterone beyond the average of 65 days. A longer period of prolonged luteal function would be very beneficial in the application of methods of estrus suppression. More research to extend prolonged CL function must be done. The results of this study help to understand the changes in the uterine environment during the state of prolonged CL function.

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